

THE BEHAVIOUR OF NATURAL KILLER LYMPHOCYTES IN PATIENTS WITH  
BENIGN AND MALIGNANT DISEASE UNDERGOING SURGERY.

C. D. M. GRIFFITH

M.D. THESIS

UNIVERSITY OF EDINBURGH

1984



"It is not, however, unlikely that the enlargement of a part containing the corculum, or first principle of cancer, may be in consequence of a process set up by the constitution for confining the effects of the disease to the part where it originally existed: thus preventing its ravages or influence upon the constitution."

*Institution for Investigating the Nature of Cancer.*

*The Edinburgh Medical and Surgical Journal 1806, 2, 389*

CONTENTS.

Declaration.	2
Acknowledgements.	3
Summary	4-5
Chapter 1. Introduction and literature review.	6-37
Chapter 2. Aims of the present study.	38-39
Chapter 3. Patients, materials and methods.	40-61
Chapter 4. Experimental results relating to the tumour effect on natural killer lymphocytes.	62-105
Chapter 5. Experimental results relating to the behaviour of natural killer lymphocytes during anaesthesia and surgery.	106-139
Chapter 6. Discussion.	140-155
References.	156-185
Publications from the work performed in preparing this thesis.	186-187

DECLARATION.

I declare that the work presented in this thesis is my own.

Some aspects of the laboratory work described were performed by other workers, their contribution is duly acknowledged.



ACKNOWLEDGEMENTS.

I wish to acknowledge the support and advice from Dr. R. C. Rees and Mr. K. Rogers during the preparation of this thesis and to thank Professor C. Potter for allowing me to use the laboratory facilities in the Virology Department of Sheffield Medical School. I am grateful to Professor R. G. Clark and Mr. K. Rogers for permission to study patients under their care and also to the patients themselves who took part in the study after giving informed consent. The research project was approved by the local hospital ethical committee.

Some aspects of the laboratory work were performed by other workers and I wish to thank Mr. A. Platts for performing many of the whole blood cytotoxicity assays and monoclonal antibody studies, Miss C. Penning for undertaking the Raji cell immunoassay for immune complexes in the plasma of cancer patients, Mr. A. Jermy for performing the assays for interferon in the plasma samples from these patients and Mrs. J. Peel for the total and differential white blood cell counts.

Finally I wish to thank my wife who gave support during the preparation of this thesis and the Department of Medical Illustrations at the Northern General Hospital, Sheffield for the illustrations.

I have been awarded a research grant from the University of Sheffield Medical Research Fund for the purchase of monoclonal antibodies used in this study. Much of the equipment in the Virology Department used in this study was supplied by the Yorkshire Cancer Research Campaign.

The Behaviour of Natural Killer Lymphocytes in Patients with Benign and Malignant Disease Undergoing Surgery.

Summary.

The natural killer lymphocyte is of interest to both the cancer researcher and cancer surgeon because of its likely role in immune surveillance and defence against developing tumours and metastatic spread of tumours.

In this study, the influence of established tumours of the stomach and colon was studied with respect to the capacity of the natural killer lymphocytes of the tumour bearing host to kill the K562 tumour target cell line. Patients with localised malignant disease had natural killer lymphocyte activity within the range of normal benign controls, however those patients with advanced tumours and metastatic spread, displayed grossly depressed levels of natural cytotoxicity. The passage of NK lymphocytes through the tumour circulation did not significantly alter NK cytotoxicity to K562 targets and NK lymphocyte numbers as identified by the Leu 7 monoclonal antibody were not reduced after passage through the tumour circulation. Plasma from the peripheral venous blood of tumour bearing patients was found to inhibit the natural killer lymphocyte activity of normal controls to kill K562 target cells; this effect was observed to a much greater degree, however, when control lymphocytes were exposed to plasma from venous blood draining the tumour circulation. This strongly suggests the production of factors by the tumour or tumour infiltrating host cells which inhibit the host natural killer lymphocyte response to tumour cells.

Surgical resection of a malignant tumour under general anaesthesia remains the main hope of curing the disease therefore the behaviour of natural killer lymphocytes in patients with cancer during anaesthesia and surgical operation was studied. The cytotoxicity of host natural killer lymphocytes to tumour target cells was enhanced during surgery in those patients with localised malignant tumours and benign controls, however patients with metastatic disease did not show this phenomenon. The observed enhancement of natural cytotoxicity occurred during the surgical procedure under general anaesthesia but not during anaesthesia alone, and was found to be an interferon independent phenomenon with the elevated cytotoxicity correlating well with an absolute rise in the Leu 7 positive (NK) lymphocytes in the circulating blood during surgery. Natural cytotoxicity to tumour target cells was further enhanced in vitro by exposure of host lymphocytes to human leucocyte  $\alpha$  interferon. There may, therefore, be a place for selective immunological enhancement of the host antitumour mechanisms such as the natural killer lymphocyte system by agents such as interferon in the perioperative period. Patients undergoing surgical resection of a malignant tumour have large numbers of circulating malignant cells which can implant to form metastases and stimulation of host defences during the perioperative period may result in reduction or abolition of metastatic disease.

CHAPTER 1.

## Introduction.

- 1.1 Cellular growth control mechanism - immune surveillance.
- 1.2 Mechanisms of the immune response to tumours.
- 1.3 Neoantigens on animal and human tumours.
- 1.4 Humoral immunity against tumours.
- 1.5 Cellular immunity against tumours.
- 1.6 History of natural killer cells.
- 1.7 Characteristics of natural killer cells.
- 1.8 Function of natural killer cells.
- 1.9 Evidence of natural killer cells as effector mechanisms in defence against tumours.
- 1.10 Natural killer lymphocyte activity and patient survival.
- 1.11 Natural killer cell activity in prevention of tumour metastases.
- 1.12 Modulation of natural killer cell activity.
- 1.13 Intra-tumour natural killer cell activity.
- 1.14 The effect of anaesthesia and surgery on immune mechanisms.
- 1.15 The effect of anaesthesia and surgery on NK cell activity.

## Introduction.

A malignant tumour develops because of the uninhibited growth of abnormal or transformed host cells which have escaped from the normal homeostatic mechanisms which control cell growth and mobility. Cells from malignant tumours have the capacity to multiply indefinitely until they have outgrown their supply of nutrients or have been destroyed by drugs, irradiation or surgical removal of the tumour mass. Malignant cells from solid tumours do not possess the normal intercellular adhesive properties which limit cell mobility and unlike cells from a benign tumour, malignant cells are able to penetrate endothelial lined surfaces and hence establish direct extensions of tumour in lymphatic and vascular channels capable of releasing tumour cell emboli which after implantation form distant tumour metastases in lymph nodes and organs such as liver, lung and bone.

The breakdown of the normal cellular growth control mechanisms and the response of the host to the developing tumour have been the subject of intense scientific interest in both experimental tumours and those arising in the human host. The study of animal tumour models which like human malignant tumours metastasize spontaneously, will aid in the understanding and future treatment of patients with cancer.

### 1.1 Cellular growth control mechanisms - immune surveillance.

A multicellular organism like man requires a mechanism to control uninhibited cell growth and eliminate cells that are faulty and may ultimately harm the existence of the host. An early concept of such a mechanism was that of allogenic inhibition conceived after

Bergheden and Hellström (1966) reported that mouse tumour cells of different histocompatibility types from the host failed to grow in contact with these cells. Klein (1966) proposed that control of cellular integrity in a multicellular organism was as likely to be due to allogenic inhibition as a surveillance mechanism based on immunological mechanisms, however the main evidence against this proposal is that allophenic mice, namely mice with cells of 2 genetically different histocompatibility types with different cell surface antigens can exist. (Mintz and Silvers, 1965).

Thomas (1959) suggested that "it is a universal requirement of multicellular organisms to preserve uniformity of cell type ... the phenomenon of homograft rejection will turn out to represent a primary mechanism for natural defence against neoplasia". With the discovery of new antigens in tumours arising in pure line mice (Foley 1953; Prehn and Main 1957), Burnet (1970) conceived that it was possible that small accumulations of tumour cells could develop but because of the tumour specific antigens expressed by these cells, they would provoke an effective immunological reaction with subsequent regression of the tumour. This system of surveillance and tumour lysis is believed to have arisen in vertebrate animals as a defence against early death from tumours developing from cells with malignant potential. These particular cells could arise as a result of aberrant division of a single cell with the subsequent formation of a malignant clone of cells which would multiply thus ultimately forming a tumour.

The most primitive vertebrate creature living today is the lamprey and it has been studied to determine the origins of the immune system present in higher vertebrate animals including man. The lamprey has a



very poor antibody response with very low levels of circulating immunoglobulin (0.2 mg/ml) which is homologous with IgM. (Marchalonis and Edelman 1968). It does however possess an active cell mediated response to tuberculin and whilst skin autografts were accepted skin homografts gave a classical rejection response (Finstad and Good 1966). The lamprey does not possess a thymus, spleen or recognisable bone marrow, but there are collections of lymphoid cells which may have a function similar to these organs. The lamprey displays the most basic requirements for an active cell mediated immunological surveillance mechanism with:

1. Collections of lymphoid cells which are thought to be the functional equivalent of the thymus, spleen and bone marrow of higher vertebrates.
2. Circulating lymphoid cells.
3. The capacity to reject tissue homografts.
4. The ability to develop a delayed hypersensitivity reaction to antigens.

Cancer and other malignant tumours appear to be almost exclusively the fate of vertebrates since creatures of lower phylogenetic orders such as insects very rarely develop tumours. A single cell line in a multicellular organism such as man is thought to undergo a finite number of cell divisions unlike a malignant cell line which can divide indefinitely. In the average human lifespan there are  $10^{16}$  cell divisions and therefore the possibility of aberrant cell division producing cells with malignant potential is great and the requirement for a mechanism to control and eliminate abnormal cells is obvious.

There is clinical evidence in both animals and man for an active immune surveillance mechanism which can recognise abnormal cells and destroy them at a premalignant or frankly malignant stage. The commonly cited evidence (Burnet 1970) for active immunological surveillance is as follows:



1. There is an increased incidence of malignant disease at the extremes of life when immunological activity is low.
2. Depression of the thymus dependent immunological response system is associated with an increased incidence of malignant disease. (This has now been questioned with the discovery of natural killer lymphocytes in nude mice which have no thymus and the lack of tumour development in mice with normal levels of natural killer cell activity).
3. Patients who are immunosuppressed (e.g. renal transplant recipients) have a higher incidence of tumours, mainly lymphomas and skin cancers, than normal controls.
4. In human autopsies, the chance finding of malignant tumours is far higher than the expected incidence of these types of tumours during life. This finding implies that tumour formation is reasonably common but does not progress due to active restriction or lysis of tumour by a functioning immune surveillance mechanism.
5. There exists documented cases of spontaneous regression of established malignant tumours in human patients.

Everson and Cole (1966) extensively reviewed the literature of well documented human tumours which had undergone spontaneous regression. Reports such as these plus the clinical observation that many tumours appear to lie dormant for months or even years would generally support the concept of immune surveillance against malignant tumours arising in the human host. Why this system is not effective in all malignant tumours remains under intense investigation, the two most likely possibilities being:

1. That the host fails to recognise the tumour as foreign tissue.

2. That tumour recognition by the host does occur, but the host response is abrogated by tumour products or tumour directed suppression by host cells.

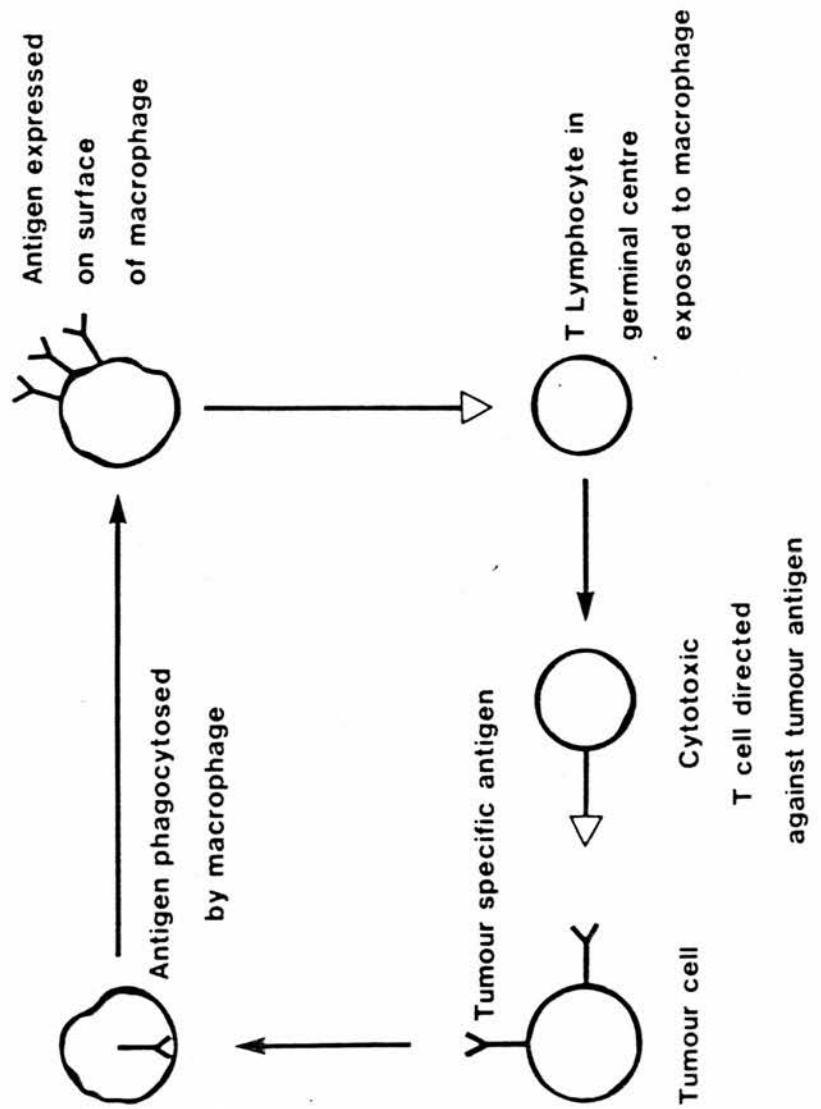
The establishment of a host response by the tumour is vital for tumour directed cytotoxic mechanisms to be activated by the host.

### 1.2 Mechanism of the immune response to tumours.

Conventional immunological theory dictates that for an immune response to be mounted by the host towards foreign or abnormal cells, whether they be invading micro-organisms or developing tumour cells, the cells must be recognised as 'non self' by the host with the detection of novel antigens on the cell surface membrane. The evidence that human tumours possess tumour specific antigens capable of stimulating an autologous host response is controversial (Hewitt 1982), (tumour specific 'antigens' such as carcinoembryonic antigen and alphafetoprotein are in fact tumour associated proteins but not antigens in the true sense). Assuming that human tumours do in fact express novel surface antigens, this antigen is processed by the reticuloendothelial system and can lead to a humoral response mediated by the B (Bursa equivalent) lymphocytes and plasma cells producing specific antibody directed towards the antigens at the cell surface with subsequent destruction of the cell by complement dependent mechanisms or by polymorphonuclear leucocytes and macrophages. Alternatively, (Fig.1) the novel tumour antigen is transported to the germinal centres in the lymph nodes and spleen by accessory cells such as macrophages. This leads to the production of a specific T (thymus dependent) cell response with the cytotoxic T cell as the main effector cell directed towards destruction of the

FIGURE 1

## PRESENTATION OF TUMOUR ANTIGEN TO T LYMPHOCYTES



tumour cell either directly, or by the actions of its cellular products (lymphokines), which can attract and then immobilise accessory cells such as macrophages and polymorphs at the site of the abnormal cells to aid in their destruction. As part of the control of the specific T cell response, suppressor lymphocytes are also generated during the immune response to a novel antigen, these cells regulate the specific T cell response and may in fact block the action of cytotoxic T cells towards the tumour cells possibly by binding with the antigen recognition site of the cytotoxic T cell. The T lymphocyte is thought to require to recognise the tumour specific antigen and major histocompatibility antigens on the tumour cell surface simultaneously to produce cytotoxic effects. This has been shown in mice with virally induced tumours (Doherty et al 1976) but remains unproven for human tumours. The antigen receptor sites on the lymphocyte are thought to be clonally distributed and therefore after recognition of the antigen by the T lymphocyte, the clone of lymphocytes with receptors for that particular antigen undergoes clonal expansion with release of T lymphocytes directed towards the antigen expressed on the tumour cell.

### 1.3 Neoantigens on animal and human tumours.

Novel tumour specific antigens distinct from the major histocompatibility antigens have been demonstrated on animal tumours. In 1943, Gross demonstrated that isografts of a chemically induced tumour were immunogenic and Prenn and Main (1957), amongst other workers showed that inbred mice with identical histocompatibility antigens have novel antigens associated with transplantable tumours.

The presence of tumour specific transplantation antigens (TSTA) can be demonstrated in vivo where prior exposure and therefore immunisation against a specific tumour stimulates immunity to subsequent tumour challenge with a tumour producing dose of live tumour cells. Tumour specific transplantation antigens have been studied extensively in animals with chemically induced tumours, particularly rodent sarcomas and carcinomas induced by polycyclic hydrocarbons. These tumours are highly immunogenic and induce a strong tumour rejection response. In these animals, immunisation by inoculation of attenuated tumour cell preparations (e.g. irradiated cells) or tumour graft resection carried out before tumour cell challenge, leads to failure of tumour development in immunised animals compared to tumour growth in control animals. This is taken to represent direct evidence of TSTA's on these chemically induced tumours. (Boyse 1963; Klein et al 1960). Tumours induced in animals with DNA viruses also express a tumour specific transplantation antigen which is common to all tumours induced by the same virus.

Spontaneously arising animal tumours however, are often either weakly or non immunogenic (Hewitt et al 1976; Middle and Embleton 1981) and these tumours are perhaps more representative of the situation in human malignant tumours (Hewitt 1982) and therefore caution is required in extrapolating the behaviour of animal tumour models which have been induced with powerful chemical agents to human tumours.

Tumour specific antigens of human tumours have been implied by means of various in vitro tests particularly the cytotoxicity assay. The work of Hellstrom (1967), using a colony inhibition assay and Takasugi and Klein (1970) with a microcytotoxicity assay was taken to show that at the cell surface of human cancer cells, individually specific and

organ specific tumour antigens were present to which the host mounted a cell mediated immune response. Subsequent work, however, has shown that peripheral blood lymphocytes show natural antitumour cytotoxicity (O'Toole et al 1973) which must therefore question the results of these original studies.

Using the above techniques and immunofluorescent staining techniques, tumour specific antigens for human tumours have been detected for melanoma (Lewis et al 1969; Shiku et al 1980), astrocytoma and renal carcinoma (Shiku et al 1980). Lymphocytes from patients with a variety of tumours have been shown to undergo blastogenesis when cultured with autologous tumour cells and also to develop cytotoxicity towards autologous tumour cells used as targets (Vanky and Argov 1980; Vose et al 1978) in cytotoxicity assays. This would suggest that the host lymphocytes are sensitised to autologous tumour cell antigens for these types of tumours. In an attempt to show the presence of human tumour specific antigens in vivo, researchers have injected tumour cell extracts subcutaneously in patients to assess delayed type hypersensitivity reaction to supposed tumour specific antigens. (Wells et al 1973; Hollinshead et al 1974). Apart from the rather dubious ethical considerations of this type of experiment, results have been difficult to interpret because of problems with bacterial contamination of tumour cell extracts and the lack of normal control tissue extracts, despite these objections however, a positive response with induration at the injection site did appear to correlate favourably with prognosis.

#### 1.4 Humoral immunity against tumours.

In general, tumour specific cytotoxic antibodies have not been detected in tumour bearing animals or humans. In rats with chemically induced



sarcoma, resection of the tumour leads to the appearance of antibodies in the serum and under laboratory conditions i.e. prolonged incubation in the presence of complement in vitro, these antibodies have cytotoxic activity.

In humans with malignant melanoma (Lewis et al 1969; Shiku et al 1980) and renal carcinoma, (Shiku et al 1980) circulating antibodies can be found in the course of the disease and following tumour excision which in vitro can lyse tumour cells in the presence of complement. Tumour specific antibody may have a role in antibody dependent cytotoxic cells (ADCC) which are a lymphocyte subset capable of killing tumour cells in the presence of such tumour specific antibody.

In experimental systems, antibody alone does not confer transplant immunity to the animal and antibody may in fact enhance tumour growth in the experimental animal (Moller 1964; Baldwin 1974), perhaps by combining with the antigen on the tumour cell surface and blocking the cytotoxic T cell mediated response.

It seems unlikely from the above evidence that tumour specific antibody alone is a major cytotoxic mechanism against tumours.

### 1.5 Cellular immunity against tumours.

The classical concept of immune surveillance against tumours involves the sensitised T lymphocyte as the effector mechanism. The cytotoxic T cell is specifically programmed to recognise cells bearing abnormal surface antigens and cause their death either directly or through the action of lymphokines which direct accessory cells such as macrophages to participate in cell killing.

The classical concept of immune surveillance with the sensitised T lymphocyte as the effector cell in the primary defence role against developing tumour cells is now subject to question after consideration of the following:

1. The delay between recognition of tumour antigen, processing of antigen and production of sensitised T cells is in the order of 7 to 10 days and there is perhaps a place for a more rapid primary defence mechanism of circulating cells which can recognise abnormal cells, bind to them and kill them within hours rather than days.
2. Experiments with T cell depleted mice (Stutman 1979) showed resistance to challenge with syngenic tumour cells but impaired rejection of allografts. Further experiments with nude mice, which are congenitally athymic and hence have an absolute lack of T cells, have failed to demonstrate an increased incidence of spontaneous tumours over normal control animals (Rygaard and Povlson 1976). This suggests the existence of a T cell independent immune surveillance mechanism which has now been identified in the mouse as the natural killer (NK) lymphocyte.
3. O'Toole and her colleagues (O'Toole et al 1973) have demonstrated using a 4 hour chromium release cytotoxicity assay that the cytotoxic cells present in patients with carcinoma of the bladder were not T lymphocytes. In retrospect it would appear that these cytotoxic lymphocytes were analagous to NK lymphocytes.
4. Perhaps the main objection to the conventional immune surveillance theory, with the tumour cells expressing tumour specific antigens leading to a specific T cell response directed against the tumour cells is, the lack of evidence to support the existence of tumour specific transplantation antigens as spontaneously arising animal and human tumours in that these tumours are non or only weakly immunogenic.



## Natural Killer Cells.

### 1.6 Historical.

Natural cytotoxicity or the ability of peripheral blood mononuclear cells to recognise and kill tumour target cells without prior processing of antigen was initially thought to be a laboratory artefact or the "background noise" of the 4 hour chromium release assay of Brummer et al (1968) used to detect specific cytotoxic activity of lymphocytes from tumour bearing individuals. Lymphoid cells from normal patients with no prior exposure to malignant disease or even to patients harbouring such disease were used as "control" lymphocytes in experiments looking for specific cytotoxicity against tumour target cells by lymphocytes from patients with leukaemia. The lymphocytes from these control patients were found to have considerable levels of cytotoxic activity against the leukaemia cells used as targets (Rosenberg 1972). A further study by Kay (1974) showed that lymphocytes from normal donors were cytotoxic to sarcoma and carcinoma cells.

This property of natural cytotoxicity is now attributed to a subpopulation of lymphocytes designated the natural killer or NK cell.

### 1.7 Characteristics of natural killer cells.

Natural killer (NK) cells have been found in the blood and spleen of rodents, birds and man. The precise lineage of the NK cell has not yet been defined completely but it appears that NK cells are a subset of lymphocytes which are distinct from mature B and T cells. Surface marker studies have been performed to relate NK cells to other lymphocyte subsets. Virtually all human NK cells express surface receptors for the Fc portion of IgG (Pross et al 1977) and 50% have low affinity receptors

for sheep erythrocytes and form rosettes (West et al 1977). NK cell activity can be obliterated by anti-T cell serum plus complement (Kaplan and Cauewaert 1978) which would support a prethymic origin for these lymphocytes and NK cells may be prethymic T lymphocytes. NK activity in rats and man is identified in the large granular lymphocyte (LGL) fraction (Timonen et al 1981), which comprises about 5% of circulating blood lymphocytes.

The NK cell may also be the same cell (killer or K cell) that mediates antibody dependent cytotoxicity. This is supported by the findings of receptors for the Fc fraction of IgG on NK cells which would enable the same cell to produce cytotoxic effects by either interacting with antibody coated target cells with the Fc receptor site or target cells with separate "NK receptors" (Koide and Takasugi 1977). Herberman and Holden (1978) have shown that some target cells sensitive to NK activity were able to inhibit antibody dependent cytotoxicity suggesting that there are 2 separate receptor populations on the effector cells.

Studies by Santoni et al (1979), and Ojo and Wizzell (1978) using nucleated target cells have shown similarity between NK and K cell types, both have a similar ontogeny, tissue distribution and strain distribution pattern. Both cell types are sensitive to in vivo cyclophosphamide treatment with depression of cytotoxicity (Herberman and Holden 1979) and both NK and K cell activity are stimulated by adjuvants such as interferon (Ojo and Wizzell 1978).

### 1.8 Function and possible role for NK cells.

Natural killer cells have been shown to be cytotoxic to tumour cells, virus infected cells and some normal cells such as bone marrow

(Herberman and Ortaldo 1981). A postulated role for these cells is that of a primary mechanism of host defence against developing tumour cells (Herberman 1981) and a secondary role in preventing metastatic spread of established tumours (Hanna and Fidler 1980).

In mice, NK activity first appears at 3 weeks of age, becomes maximal at 8 to 10 weeks and then gradually diminishes (Herberman and Ortaldo 1981). NK activity in the rat and hamster is not age restricted and in man NK activity is found in foetal cord blood and remains present to old age. Rosenberg et al (1973) have found in humans that natural killer cell activity was maximal over 16 years of age and this probably does not diminish significantly during adult life (Takasugi et al 1973). NK activity of individual donors remains remarkably constant (Pross and Baines 1982) but in females there may be slightly enhanced NK cell activity in the second half of the menstrual cycle suggesting that endocrine factors, particularly the female sex hormone oestrogen, may influence NK cell behaviour (White et al 1982). There exists a diurnal variation in T lymphocyte numbers but this is not found with NK lymphocytes (Ritchie et al 1983) as defined by the monoclonal antibody Leu 7.

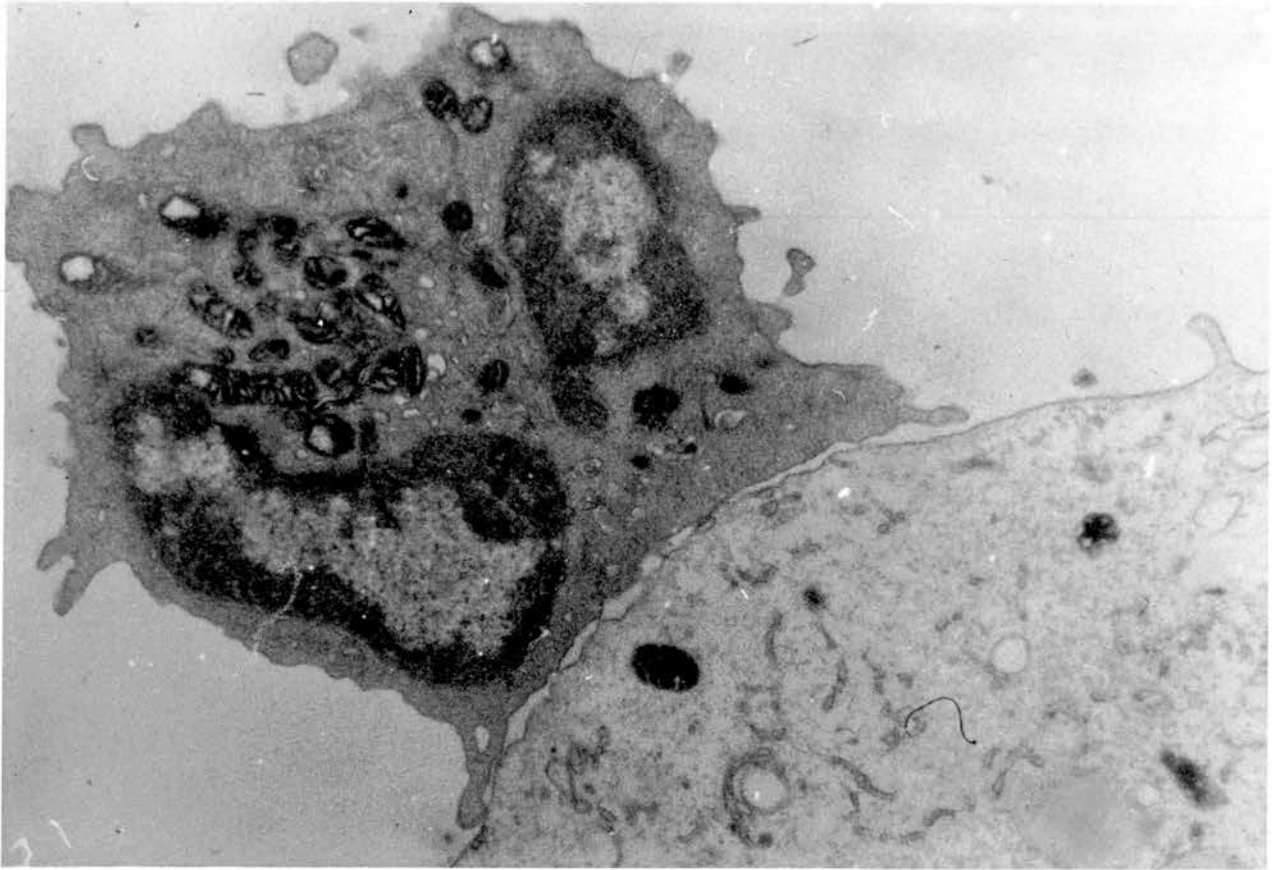
One of the main objections to the T cell playing a major role in the primary defence against developing tumour cells in humans is the considerable delay of 7 to 10 days between the initial contact with the tumour specific antigen and also recognition of the major histocompatibility complex on the surface of the tumour cell and the production of sensitised lymphocytes which are either directly cytotoxic to the tumour or promote cytotoxic activity of other cells. NK activity and target cell lysis, however, occurs within hours of exposure of NK sensitive tumour target cells to NK cells. NK sensitive cells are thought to possess NK

surface receptors at which the NK cell binds. The findings by Jondal and Targan (1978) that trypsin abolishes Nk activity is interpreted as obliteration of these sites by the action of trypsin.

Vondinelich et al (1983) have suggested that the target structure on susceptible cells which is recognised by the NK cell could be the transferrin receptor sites. These workers found that target cell lines with few or absent OKT9<sup>+</sup> cells (OKT9a is a monoclonal antibody which recognises an epitope on the transferrin receptor) were resistant to lysis by NK cells while those cell lines which carried the receptor were largely NK sensitive. NK cell recognition of target cells was inhibited with a trypsin cleaved fragment of the receptor protein with a molecular weight of 70,000 purified from an OKT9-bearing affinity column.

After recognition of the receptor site on the target cell surface the NK cell binds to the target cell by forming adhesions, (Hiserodt et al 1982), a process that takes 20 to 30 minutes and requires Mg<sup>+</sup> to form a conjugate (see Fig.2). A lytic process which requires Ca<sup>+</sup> is then activated with subsequent lysis of the tumour cell within 3 to 6 hours. The mechanism of target cell lysis after binding of the NK cell is still not clarified but may be performed by the enzymes (proteases and phospholipases) thought to be present in the granules of the LGL's which have NK activity (Goldfarb et al 1982). A protease inhibitor (phenylmethyl-sulfanyl-flouride) has been shown by Hudig et al (1981) to ablate NK activity suggesting that this enzyme may play a major role in target cell killing. Recent work by Roder et al (1982) however, has demonstrated that NK cells respond to NK sensitive target cells with a rapid burst of oxygen metabolites which are involved in NK mediated cytotoxicity and may in fact be the mechanism of cell killing.

FIGURE 2.



ELECTRON MICROGRAPH OF A HUMAN LARGE GRANULAR (NK) LYMPHOCYTE (CENTRE) BINDING  
TO A K562 TARGET CELL TO FORM A CONJUGATE.



After target cell lysis, the NK cell dissociates from the lysed cell and is capable of repeating the sequence of tumour cell recognition, binding and lysis many times, this is referred to the recycling capacity of the NK cell. Since this system of recognition and lysis of abnormal cells is active within hours of introduction of these cells it should constitute an effective mechanism in the initial phase of defence against tumour formation with the specific T cell response becoming active against the tumour at a later stage.

#### 1.9 Evidence for NK cells as effector mechanisms in defence against tumours.

There is mounting evidence that NK cells are an important part of the defence system against developing tumours in both animals and man.

The evidence for the role of NK cells in resistance to in vivo tumour growth in mice is summarised below:

1. There is a correlation between levels of NK activity and resistance of growth of NK sensitive tumours in various strains of mice.
2. Poor growth of NK sensitive tumours in nude mice (T cell deficient) as compared to euthymic mice of the same strain.
3. More resistance to growth of tumours in young mice, at the peak of NK activity, than in older mice.
4. T Cell deficient chimeras reconstituted with bone marrow from a high NK strain, have high NK activity and increased resistance to growth of NK sensitive tumours.

5. More rapid and progressive growth of NK sensitive tumours in beige mice which have no NK lymphocytes.
6. Close correlation between levels of NK activity and degree of rapid clearance, of intravenous inoculated, radio-labelled NK sensitive tumour cells, from the lungs and other organs.

Haller et al (1977) found that the growth of NK sensitive tumours in mice was influenced by the NK cell status of the recipients in that mice with high NK activity (nude mice, high NK strain, mice 5-8 weeks of age) there was a lower incidence of progressive tumour growth after tumour challenge than mice with low NK activity.

Mice subjected to lethal X irradiation followed by reconstitution with histocompatible bone marrow stem cells from low or high NK strains produced T cell deficient mice with either high or low NK activity (Haller et al, 1977). Tumour resistance in vivo was found to be directly correlated with high NK activity.

Beige mice are mice with a mutant gene (beige gene) which leads to a complete and selective impairment of natural killer cell activity but leaves the remaining forms of cell mediated immunity intact. Spleen cells from these mice, which in normal mice are rich in NK cells, failed to lyse YAC cells, a lymphoma cell line which is exquisitely sensitive to NK cells (Roder et al 1979).

The human analogue of the NK cell deficient or beige mouse exists in patients with the rare Chediak-Higashi syndrome. These patients have a selective deficit in NK activity, but humoral and delayed type hypersensitivity are normal (Roder et al 1979 ). They are known to have a high incidence of lymphoproliferative disorders which is taken to indicate a basic failure of the primary immune surveillance mechanism.

Human NK lymphocytes have been shown in vitro to have activity against tumour target cells derived from human tumours ranging from those cell lines which are exquisitely sensitive such as the K562 cell line to those which are completely resistant. The K562 cell line used in the 4 hour chromium release assay to detect cytotoxic activity of human NK cells derives from the pleural effusion of a patient with myeloid leukaemia in blast crisis. (Lozzio and Lozzio 1975).

In man, NK lymphocytes have also been demonstrated to have activity against tumour cells derived from lymphoid malignancies (Rosenberg et al, 1972) and also some solid malignancies such as carcinoma of the colon and lung (Vose and Moore 1980) malignant melanoma (Peter et al 1975, Hersey et al 1980) and ovarian carcinoma (Mantovani et al 1983). It is relevant that patients with malignant disease who have a large "tumour burden" have low or undetectable levels of NK activity in the peripheral blood (Pross and Baines 1976; Harowitz et al 1983). This probably represents depression of NK activity by the large tumour load rather than a primary immune defect involving NK cells allowing uninhibited tumour growth. Studies of fresh human tumours have attempted to demonstrate NK activity in tumour infiltrating lymphocytes however, either very low or absent NK activity is usually reported, (Vose 1980; Bland et al 1981; Becker S 1980), although studies from our laboratory have found significant natural cytotoxicity of tumour infiltrating lymphocytes (from tumours removed from patients studied in this thesis) in about 30% of colorectal tumours studied (Clegg 1983).

#### 1.10 Natural killer lymphocyte activity and patient survival.

The results of some studies into human NK lymphocyte activity have suggested a correlation between levels of NK activity and subsequent

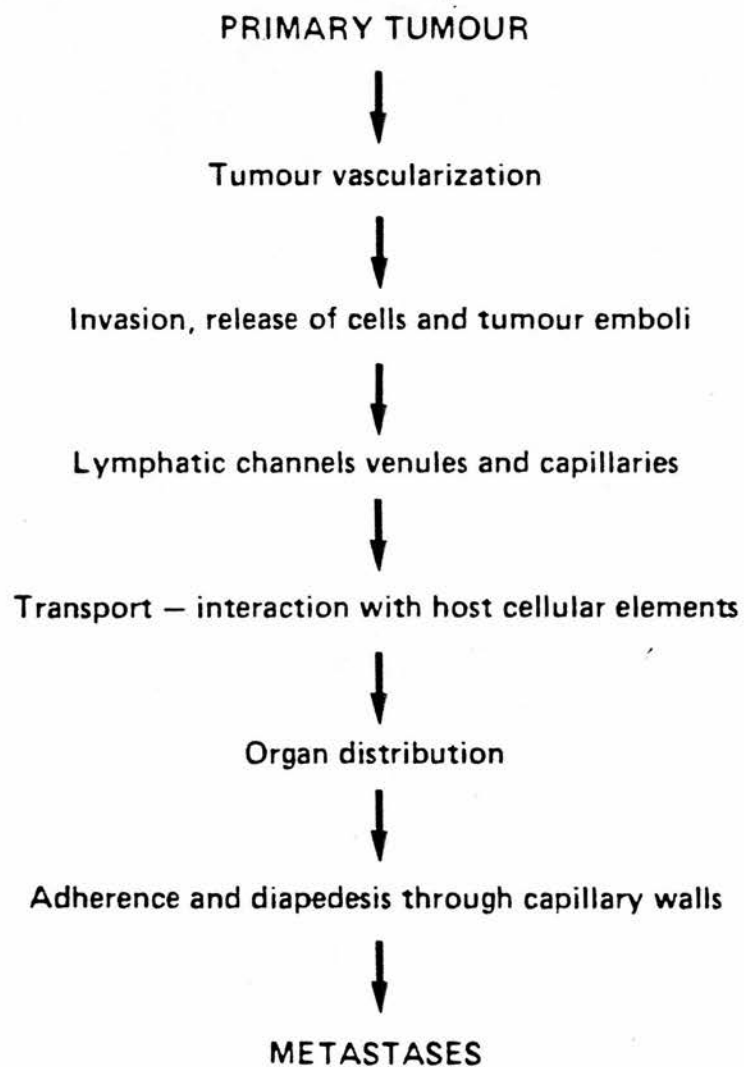


prognosis. In patients with malignant melanoma, those patients with high levels of NK activity following removal of local recurrent tumour developed significantly fewer distant metastases than those with low NK activity. (Hersey et al 1982). NK activity in these patients appeared also to correlate with thickness of the tumour which is the main factor in determining the prognosis of these patients. Other studies also suggest some correlation between host survival and host levels of NK activity although the evidence for this is not totally consistent (Karre et al 1980; Gorelik et al 1981) and possibly natural resistance to tumours as mediated by NK lymphocytes is a heterogenous phenomenon.

#### 1.11 NK cell activity in prevention of tumour metastases.

The natural killer lymphocyte in addition to T cells and macrophages may be an effective defence against intravascular tumour metastases even when the tumour cells have overcome the natural resistance mechanisms which have failed to prevent its primary growth. Cells from a malignant tumour have the capacity to invade endothelial lined surfaces such as veins and lymphatic channels with the release of tumour cells into the venous and lymphatic circulations which can then form distant tumour metastases. (See Fig.3). Haematogenous metastases form when circulating emboli of tumour cells impact in a capillary circulation such as the liver or lung, the preferential site for tumour cell arrest is usually the first capillary circulation reached after initial venous invasion. Clumps of tumour cells rather than individual cells are most likely to form metastases (Liotta et al 1976) which become covered with a sheath of fibrin from fibrinogen in the blood, then invasion of the capillary wall occurs with the subsequent development after tumour cell replication of a distant tumour metastases (Wood 1956). A primary tumour, however,

Fig. 3



A diagrammatic representation of the development of the primary tumour, its dissemination, and the development of metastases.

(after WOOD, 1958)

may be kept dormant and localised if the tumour cell emboli can be lysed by circulating macrophages, activated T cells or NK cells.

Riccardi et al (1979), were the first to show that in mice there was an effective clearance of radio labelled tumour cells from the lungs and other organs within 2 hours of intravenous inoculation, this time scale is too short for macrophages or other lymphoid cells to be effective and therefore the authors concluded that the NK cell was the responsible effector cell in this situation, this premise is strengthened by the fact that the rapid clearance from the lungs was related to the pre-existence of NK cells in that organ.

Hanna et al (1980) confirmed that clearance of radio labelled tumour cells correlate with NK activity and also that tumour resistance of cyclophosphamide treated mice (which have depressed NK activity) can be restored by systemic transfer of spleen or bone marrow cells which repopulate the mice with NK cells.

It is of interest therefore that beige mice which have a selective deficit in NK activity had increased metastatic spread of Lewis lung tumour when implanted subcutaneously (Salomen et al 1980) yet nude mice which are T cell deficient but possess normal NK activity did not develop tumour metastases. (Freedman et al 1976).

In humans, the role of NK cells in the prevention of haematogenous tumour metastases is unknown but the requirement for an effective intravascular defence system is self evident. The finding of tumour cells in the circulating blood, particularly during surgical manipulation of the tumour, is well documented (Salsbury et al 1965; Roberts et al 1960) and the effect of anticoagulants in reducing metastases (El Rifi et al 1965) may operate by delaying arrest and implantation of tumour cells

by preventing the formation of a fibrin coat such that the intravascular tumour defence systems (NK cells, T cells and macrophages) have more time to eliminate tumour cell emboli. If NK cells do have a role in prevention of metastases in man, patients whose NK activity is depressed in some way by a large tumour load may be more susceptible to subsequent development of distant haematogenous metastases.

#### 1.12 Modulation of NK activity.

NK cells are similar to other biological systems in that they are subject to factors which enhance NK activity as well as inhibiting factors which control the overall activity of these cells.

##### Enhancement of NK activity. Interferon and interferon inducers.

Interferon was first identified by Isaacs and Lindenmann in 1957. The interferons are now known to be a family of glycoproteins ( $\alpha$ ,  $\beta$  and  $\gamma$ ) which are synthesised by cells in response to viral infection, immune stimulation and a variety of chemical inducers. The interferons have been found to have a wide range of biological activity apart from their antiviral action including inhibition of tumour cell multiplication (Gesser 1977), enhanced lymphocyte cytotoxicity (Heron et al 1976), and macrophage activation (Rabinovitch et al 1977) which has lead to their use as a potential antitumour agent.

Interferon is the most potent known stimulator of natural killer cell activity (Herberman et al 1979) and known inducers of interferon such as bacterial adjuvants (BCG), viruses, tumour cell lines (Djeu et al 1980) and chemical agents such as polynosinic acid - polycytidytric acid (poly 1:C) (Zarling et al 1980) can also enhance NK activity.

Interferon boosts NK activity in rodents and man by recruitment of NK cells, possibly pre NK cells to mature cells with cytolytic activity, and increasing the kinetics of target cell lysis by improved binding to target cells (Stewart et al 1982) and increased recycling ability of effector cells (Ullberg et al 1981) to enable them to detach from the lysed cell and bind and kill further target cells. Interferon inducers act by stimulating interferon production by macrophages or lymphocytes: in the mouse there is an absolute requirement for monocytes to be present for enhancement of NK activity to occur, but in man however certain interferon inducers such as poly IC are able to enhance NK activity without accessory macrophages in vitro, (Koren et al 1981) possibly by direct stimulation of NK cells to produce interferon. Addition of anti interferon antibodies to human and animal test systems causes a failure of enhancement of NK activity.

Large tumour burdens (Pross and Baines 1976; Kadish et al 1981) and severe protein calorie malnutrition (Salimonu et al 1982) in man is associated with a refractory enhancement response to exogenous interferon and this may be a factor in tumour dissemination and perhaps increased risk of infection in these patients.

### Inhibition of NK activity.

#### Direct inhibition.

Prostaglandins (PG's) cause marked depression of NK activity in vitro (Salimonu et al 1982) when added to the test systems used to detect cytotoxic activity of NK cells although the mode of action is unknown.

Brunda et al (1980) examined the effect of prostaglandins on murine natural killer cell activity and found that  $\text{PGE}_1$ ,  $\text{PGE}_2$ ,  $\text{PGA}$ , or  $\text{PGA}_2$  were inhibitory to NK activity but  $\text{PGB}_1$ ,  $\text{PGB}_2$ ,  $\text{PGF}_{1\alpha}$  or  $\text{PGF}_{2\alpha}$  did not cause significant inhibition. Further support that some PG's are inhibitory to NK cells comes from the work of Koren et al (1981), who showed that monocytes incubated with poly I.C. produce PG's and inhibit NK activity but this effect was reversed by adding indomethacin, a known prostaglandin synthetase inhibitor, to the test system.

It is well established that malignant tumours of the breast (Bennett et al 1977) and lung (Bennett et al 1982) contain high levels of prostaglandin like material compared to normal breast or lung tissue. The level of this substance correlates directly with tumour invasion and spread and could be seen as an inhibitory mechanism to the immune defence role of the NK lymphocyte. Corticosteroids, some chemotherapeutic agents (e.g. cyclophosphamide) and X irradiation are also known to suppress or ablate NK cell activity. (Hochman et al 1979; Djeu et al 1979; Oehler et al 1978).

#### Inhibition by suppressor cells.

Suppressor cells induced by various treatments have been shown to depress NK activity although the type of cell responsible is variable. Suppression is not seen in nude mice suggesting that the suppressor activity in mice is mediated by suppressor T cells, (Savary and Lotzova 1978). Suppressor cells induced in mice by pyran copolymer have macrophage type characteristics in that they are capable of phagocytosis. (Santoni et al 1979).

In humans, suppressor cell activity to NK cells has been shown in malignant pleural effusion (Uchida and Michsche 1981) and also after



anaesthesia and surgery in patients with breast cancer (Uchida et al 1982). This effect was thought to be mediated by suppressor monocytes.

Prostaglandins (PG's) produced primarily by mononuclear phagocytic cells (Kurland and Bockman 1978; Kennedy et al 1980) are known to be potent inhibitors of NK activity and they are thought to be the likely mechanism of inhibition of NK activity by suppressor cells.

#### Sequestration of NK cells.

NK cells may be sequestered in tumours which could account for the depression of NK activity seen in many tumour bearing patients, particularly those with advanced malignancy. Studies of tumour infiltrating lymphocytes (TIL's) of both animal and human tumours have failed to demonstrate significant levels of NK activity (Vose 1980; Bland et al 1981; Becker 1980) and many studies have found NK activity to be totally absent in lymphocytes isolated from tumours although recent work in our laboratory has shown significant NK activity in TIL's in 30% of human colorectal tumours studied. The possibility still remains however that NK cells are sequestered in the tumour but their activity is inhibited by products from the tumour cells per se or other intratumour cells such as suppressor cells.

#### Reduction of recycling capacity.

The ability of an NK cell to bind to and cause target cell lysis then release and bind to subsequent tumour target cells is called the recycling capacity of the cell. This has been shown by kinetic studies to be reduced in patients with advanced cancers (Steinhauer et al 1982) and may be yet another factor in the reduced cytotoxic ability of NK cells found in patients with advanced malignant disease.

### 1.13 Intratumour NK activity.

It might be expected that if NK cells play a prominent early role in immune surveillance and defence against developing tumours, NK activity would be found in lymphocyte populations isolated directly from tumours. In many studies, however, NK activity has not been detected in tumour infiltrating lymphocytes in humans although NK activity in the circulating blood lymphocytes is within the normal range for patients without malignant disease. It has been proposed that defective NK activity of TIL's is due either to suppressor cells or factors operating on the TIL's or a low number of effector cells in the tumour substance (Introna et al 1982). It is perhaps not unexpected in that for a tumour to develop to such a size that it requires surgical resection, the immune defences have failed and the NK cell response has been overwhelmed. NK cells in this situation may still have considerable importance in the role of prevention of metastatic spread of the tumour by recognising and destroying tumour cell emboli.

The possible mechanisms for direct intratumour suppression of NK activity are thought to be either the generation of prostaglandins by intratumour host cells (suppressor cells) or perhaps even the tumour cells per se. Golub et al (1982) using a single cell assay against K562 targets has shown that low activity of TIL's to tumour target cells was not due to low numbers of effector cells but due to the effect of suppressor substances.

Further research is required to define the precise mechanisms of intratumour NK suppression.



#### 1.14 The effect of anaesthesia and surgery on immune systems.

The majority of patients with malignant disease of the gastrointestinal tract have exposure to a general anaesthetic and a surgical procedure either for exploratory purposes or excision of the primary tumour.

Evidence from experimental animal systems (El Rifi et al 1965; Gottfried et al 1961) suggests that either anaesthesia or the surgical stress of the operation can be associated with dissemination of the primary tumour and development of metastases, this has also been shown for human patients (Gordon-Taylor 1948). The mechanism of tumour dissemination does not appear to be entirely due to release of tumour cell emboli during surgical manipulation of the tumour (Salsbury et al 1965; Roberts et al 1960) but also due to an observed depression of the overall immune response.

There is a decrease in circulating T and B lymphocytes following anaesthesia and surgery (Slade et al 1975) with reduced lymphocyte responsiveness to mitogens and antigenic stimulation. Delayed hypersensitivity reactions are reduced or abolished following surgery indicating a basic depression of the cell mediated immune response (MacLean 1979). Cytotoxicity of lymphocytes to autologous tumour target cells is decreased post operatively in patients with breast cancer (Vose and Mougdil 1975) and malignant melanoma (Cochran et al 1973) and antibody dependent cytotoxicity is also decreased post operatively in previously immuno suppressed cancer patients (McCredie et al 1979). Neutrophil chemotaxis and phagocytosis are also reduced after surgery but return to normal levels within a few days of the surgical operation.

In order to dissect the precise relationship of anaesthesia and surgery to this depression of immunity, in vitro studies of commonly used

anaesthetic agents have shown a slight reduction of the phytohaemagglutinin response (a known mitogen) of lymphocytes and the leucocyte migration inhibition test. The main immune suppression however would appear to be due to the stress of the surgical procedure with for example the degree of depression of the lymphocyte response to phytohaemagglutinin being proportional to the degree of surgical stress (Cullen and Van Belle 1975). In vitro responsiveness to phytohaemagglutinin was found to be more depressed in cancer patients than patients with benign disease following operation.

The immune depression seen after surgery is very similar to that seen after severe trauma or burns and would therefore appear to be related to the stress response to surgery. Release of corticosteroids, catecholamines and prostaglandins occurs after surgical stress and trauma and these agents may cause immune depression. Suppressor cells have also been found in mice (Waugh et al 1982) and human patients following operation (Baker et al 1979; Uchida et al 1982). These cells have macrophage activity and could inhibit the specific T cell response and NK cell activity.

#### 1.15 The effect of anaesthesia and surgery on NK cell activity.

Uchida et al (1982) have shown a decrease in NK activity as measured by the 4 hour chromium release assay using K562 target cells in female patients with breast carcinoma following mastectomy. The NK status of these patients before surgery was comparable to benign controls. The depression of NK activity occurred 2 to 3 days following surgery and appeared to be mediated by suppressor monocytes in that 24 hour pre culture of patients cells with NK cells from normal controls suppressed the activity of these cells.

The response of patients NK cells to anaesthesia per se and the response during the period of surgical operation remains unknown.

TO:

Miss S M Mackay  
Faculty of Medicine Office  
Teviot Place  
Edinburgh EH8 9AG

I acknowledge receipt of one copy of the thesis entitled:

"The behaviour of natural killer lymphocytes in patients with benign  
and malignant disease undergoing surgery"

presented by Mr C D M Griffith for the degree of MD

Signature \_\_\_\_\_ Date \_\_\_\_\_

### AIMS OF THE PRESENT STUDY.

The purpose of the study is to determine the effect of malignant tumours in the gastrointestinal tract on natural killer cell activity of the human host. The primary treatment modality of these patients is surgical which entails an operative procedure under general anaesthetic; therefore the behaviour of natural killer lymphocytes in patients undergoing general anaesthesia and surgical operation will be studied and compared to a control group of patients with benign disease also undergoing general anaesthesia and surgical operation. Surgery remains the main hope of cure for most patients with gastrointestinal malignancy, and it can be proposed that enhancement of natural cytotoxic mechanisms, such as those mediated by the NK cell, may help reduce tumour metastases and hence improve patient survival. The specific questions that this study proposes to answer are:

1. Do malignant tumours of the gastrointestinal tract directly modify natural killer cell activity, and if so, what is the likely mechanism?
2. What is the effect of general anaesthesia and surgery on the cytotoxic potential of natural killer lymphocytes? Does the type and route of anaesthesia influence natural killer cell activity of peripheral blood lymphocytes?
3. Is there any likely therapeutic manoeuvre which can enhance natural killer cell mediated cytotoxicity to tumour cells in the intra-operative period when it is known that large numbers of tumour cells from the primary tumour are present in the circulation?

CHAPTER 3.



## PATIENTS, MATERIALS AND METHODS.

### 1. Patient Details.

### 2. General Laboratory Equipment.

- i. Chemicals
- ii. Deionised and glass distilled water
- iii. Glassware
- iv. Plastic and disposable hospital and laboratory equipment

### 3. Stock Solutions.

- i. Heparin
- ii. Lymphocyte separation medium
- iii. New born and foetal calf serum
- iv. Trypan blue
- v. Crystal violet
- vi. Phosphate buffered saline
- vii. Trypsin
- viii. Versene
- ix. Human IgG (Cohn Fraction II)
- x. Iodogen
- xi. Staphylococcal protein A

### 4. Interferon Preparation.

5. Tissue Culture.

- i. Media - RPMI 1640
- ii. Maintenance medium
- iii. Medium 199
- iv. Sterility checking
  - a. Thioglycolate broth
  - b. Sterility control
- v. Tumour, cell line - K562
- vi. Raji cell line
- vii. Maintenance of tissue culture - stationary suspension cultures
- viii. Viable cell counts
- ix. African Green Monkey kidney cells
- x. Baby hamster kidney cells

6. Semliki Forest Virus Culture.

Maintenance of viral culture

7. Monoclonal Antibodies.

8. Experimental Methods.

- i. Blood sampling
- ii. Transport of blood and tumour samples
- iii. Anaesthetic techniques
- iv. Lymphocyte separation from whole blood

8. Experimental Methods Continued.

- v. 4 hour  $^{51}\text{Cr}$  release cytotoxicity assay
  - a. Labelling of tumour cells
  - b.  $^{51}\text{Cr}$  release assay for separated lymphocytes
  - c.  $^{51}\text{Cr}$  release assay for whole blood
- vi. Preparation and storage of plasma samples
- vii. Addition of plasma samples to control PBL's
- viii. Heat treatment of plasma samples
- ix. Monoclonal antibody characterisation of leucocytes
- x. Morphological characteristics of leucocytes
- xi. Raji cell assay for immune complexes
- xii. Interferon assay
- xiii. In vitro incubation of PBL's with exogenous interferon

TABLE I (a)

Details of patients with malignant disease of the large bowel.

<u>Patient</u>	<u>Age</u>	<u>Sex</u>	<u>Tumour</u>	<u>Site</u>
JC*	58	M	Adenocarcinoma	Colon
RR*	72	M	Adenocarcinoma	Rectum
FO*	86	F	Adenocarcinoma	Colon
GW*	80	M	Adenocarcinoma	Colon
DG*	58	M	Adenocarcinoma	Rectum
JB*	69	M	Adenocarcinoma	Colon
WW*	71	M	Adenocarcinoma	Colon
FW*	88	M	Adenocarcinoma	Colon
NF*	84	F	Adenocarcinoma	Colon
WD*	65	M	Adenocarcinoma	Colon
DP*	55	M	Adenocarcinoma	Colon
MS*	65	F	Adenocarcinoma	Colon
NW*	82	F	Adenocarcinoma	Colon
AH*	65	M	Adenocarcinoma	Colon
AP*	63	M	Adenocarcinoma	Colon
GH*	59	M	Adenocarcinoma	Colon
EM	77	F	Adenocarcinoma	Rectum
JH	60	F	Adenocarcinoma	Rectum
HC	63	M	Adenocarcinoma	Rectum

\* Aortic, tumour draining venous blood and venous blood from adjacent bowel sampled.

TABLE I (b)

Details of patients with malignant disease  
of the stomach.

<u>Patient</u>	<u>Age</u>	<u>Sex</u>	<u>Tumour</u>
PW*	32	M	Lymphoma
JC*	57	M	Adenocarcinoma
WN*	52	F	Adenocarcinoma
FP*	71	M	Adenocarcinoma
BR*	69	F	Adenocarcinoma
HW*	80	M	Lymphoma
AC	67	M	Adenocarcinoma
HH	71	M	Adenocarcinoma
NF	60	M	Adenocarcinoma
FH	64	M	Adenocarcinoma

\* Aortic, tumour draining venous blood and  
venous blood from adjacent stomach sampled.

TABLE II.

Details of patients with benign disease.

<u>Patient</u>	<u>Age</u>	<u>Sex</u>	<u>Diagnosis</u>
MB*	67	M	Gallstones
NT*	68	F	Gallstones
GH*	57	M	Duodenal ulcer
GB*	40	M	Duodenal ulcer
EB*	66	M	Gallstones
MG*	41	F	Gallstones
WW*	22	M	Duodenal ulcer
PB*	34	F	Gallstones
MT*	71	F	Gastric ulcer
FA*	68	F	Gastric ulcer
DS	51	M	Colostomy
KN	28	M	Colostomy
MS	62	F	Gallstones
BI	57	F	Benign weight loss
AJ	43	F	Gallstones
MH	55	F	Gallstones
CS	65	F	Gallstones
MB	60	F	Gallstones
MC	80	F	Villous adenoma rectum
MS	64	M	Gallstones

\* Aortic,colon and stomach draining venous blood samples



## 1. PATIENTS, MATERIALS AND METHODS.

### Patients.

The study group consisted of 29 patients with histologically proven malignant tumours of the stomach or large bowel undergoing either resection of the primary tumour or exploratory laparotomy. In 22 patients it was possible to sample blood from veins directly draining the tumour. 19 patients, with mainly benign upper abdominal conditions (gallstones, duodenal ulceration) requiring surgical operations were also studied. In 10 of those patients aortic, stomach and colon draining venous blood was sampled. Patient details for both groups are given in Tables I (a), I (b) and II.

Informed consent was obtained from each patient prior to study and the protocol for the study was approved by the local hospital ethical committee.

## 2. MATERIALS AND METHODS.

### General Laboratory Equipment.

#### i. Chemicals.

General laboratory reagents of ANALAR grade were used throughout the laboratory part of this study.

#### ii. Deionised and Glass Distilled Water.

This was prepared by passing water directly from the main supply through an elgastat deionisation unit with a specific conductivity of  $>1.0 \times 10^6$  ohms/cm<sup>3</sup>. Tissue culture media preparation and most other solutions were prepared from 500 ml volumes of glass

distilled water which was sterilised by autoclaving at 20 psi for 20 minutes.

iii. Glassware.

Pyrex glassware was used which was cleaned by boiling and washing in a commercially available detergent. It was then rinsed several times with tap water followed by deionised water. All glassware was dried in a hot air oven at 80°C and then sterilised in a hot air oven at 160°C for 2 hours.

iv. Plastic and Disposable Hospital and Laboratory Equipment.

Plastic syringes (10 ml, 20 ml and 60 ml volumes) and disposable needles (25G and 19G) were used for the hospital collection of blood samples. These were obtained by the hospital pharmacy from Becton Dickinson and Company, Middlesex, England. Plastic universal containers (30 ml), plastic centrifuge tubes and plastic bijoux were purchased from Sterilin Laboratories, Middlesex, England. Round bottomed microwell test plates with 96 wells/plate were obtained from Falcon Products, Becton Dickinson and Company Ltd., Middlesex, England.

3. STOCK SOLUTIONS.

i. Heparin.

Heparin (1000 units/ml) was used to "lubricate" the plastic syringes used for venepuncture and was added to the blood samples at 0.1 ml per 10 ml of whole blood to prevent clotting during transport and subsequent assay of cytotoxicity. This was obtained by the hospital pharmacy from Weddel Pharmaceuticals Ltd., London, who also supplied the laboratory with heparin at a concentration

of 25,000 unit/ml, which was diluted to give a final concentration of 100 units/ml. Heparin was stored at 4°C until required.

ii. Lymphocyte Separation Medium (LSM).

This was obtained from Flow Laboratories Ltd., Irvine, Scotland. LSM is an aqueous solution consisting of 5.7 g of a high density sucrose - epichlorohydrin polymer (Ficoll 400<sup>R</sup> Pharmacia Fine Chemicals) and 9 g of sodium diatrizoate per 100 mls, giving a density of  $1.077 \pm 0.001$  g/ml and an osmolality of 270-300 mOsm/kg. It was stored in the dark at room temperature.

iii. Newborn Calf Serum (NBCS).

This virus and mycoplasma free serum was supplied by Flow Laboratories Ltd., Irvine, Scotland and stored at -20°C until required. Heat inactivation was performed by leaving the thawed serum in a 56°C water bath for 40 minutes.

iv. Trypan Blue.

Trypan blue powder was purchased from BDH Limited, Poole, England. A solution of one per cent was prepared in phosphate buffered saline and the excess solid residue was filtered off. The clarified solution was dispensed in 2-3 ml volumes, autoclaved at 10 psi and stored at 4°C.

v. Crystal Violet.

This was purchased from BDH, Poole, Dorset. A 1% solution by weight was prepared in phosphate buffered saline and stored at room temperature.

vi. Phosphate Buffered Saline (PBS).

This was prepared from tablets supplied by Oxoid Ltd., London.

The solution conformed to the formula described by Dulbecco et al 1954. The solution was sterilised by autoclaving at 10 psi for 10 minutes and stored at room temperature.

vii. Trypsin.

Trypsin solution was prepared by dissolving 2.5 gm of trypsin (Difco 1:250) in one litre of PBS and stirred with a magnetic stirrer at room temperature. The clarified solution was then sterilised by filtration through a Seitz filter, dispensed into 10-20 ml aliquots and stored at  $-20^{\circ}\text{C}$ .

viii. Versene.

The di-sodium salt of ethynal diamine-tetracetic acid (versene) was added to PBS at a concentration of 0.02% and the pH adjusted to pH 7.2 - 7.4 with N10 sodium hydroxide. The solution was dispensed in 10 ml aliquots, autoclaved at 10 psi for 10 minutes and stored at  $4^{\circ}\text{C}$ .

ix. Human 1 gG (Cohn Fraction II).

This was obtained in saline (20mg/ml) from Flow Laboratories, Irvine and freed of aggregates by centrifugation at  $100,000 \times G$  for 90 minutes. The protein content was adjusted to 6.5 mg/ml of saline and the solution stored in aliquots of 0.2 ml at  $-70^{\circ}\text{C}$ .

x. Iodogen.

This was supplied as Iodogen in 1 gm quantities by Pierce Chemical Company, Rockford, Illinois, U.S.A.

xi. Staphylococcal Protein A.

This was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, as Protein A in 5 mg freeze dried packs.

4. INTERFERON PREPARATION.Human Lymphoblastoid (Namalva) Interferon (HIF).

A vial of freeze dried material (batch 4656/6) of human leucocyte  $\alpha$  interferon was donated by Drs. Fautes and Johnston, Wellcome Research Laboratories, Beckenham. The vial contained approximately  $6 \times 10^5$  interferon units of a specific activity of  $6 \times 10^5$  units/mg of protein. The freeze dried interferon was dissolved in 1 ml of glass distilled water and then diluted down further to be stored at a concentration of 20,000 units HIF/0.1 ml vial at  $-80^\circ\text{C}$ .

5. TISSUE CULTURE.i. Media - RPMI 1640

RPMI 1640 (10 x concentrate) minus L-glutamine was supplied by Gibco-Biocutt (Europe) Glasgow, Scotland and made up as follows.

RPMI 1640 (10 x concentrate)	50.0 ml
Calf serum	50.0 ml
Sodium bicarbonate (4.4%)	10.0 ml
1M Hepes buffer	10.0 ml
200 mM L-glutamine	10.0 ml
Glass distilled water	355.0 ml
Antibiotics : Penicillin	0.5 ml
Streptomycin	0.5 ml



ii. Maintenance Medium.

This was prepared using minimal essential medium concentrate from Flow Laboratories Ltd., Irvine, Scotland.

Maintenance medium (10 x concentrate)	50.0 ml
Calf serum	20.0 ml
Glass distilled water	450.0 ml
Antibiotics : Penicillin	0.5 ml
Streptomycin	0.5 ml

iii. Medium 199.

This was prepared using medium 199 concentrate from Flow Laboratories Ltd., Irvine, Scotland. It was made up according to the following formula.

Medium 199 (10 x concentrate)	50.0 ml
Glass distilled water	450.0 ml
Sodium bicarbonate solution (7.5%)	5-10ml
Antibiotics : Penicillin	0.5 ml
Streptomycin	0.5 ml

iv. Sterility Checking.

- a. Thioglycolate Broth. This broth was prepared by dissolving one tablet (Oxoid Limited, Hampshire) in 10 ml of deionised water, the solution was then autoclaved at 15 psi for 15 minutes and kept at room temperature until required for sterility monitoring of all tissue culture media.
- b. Sterility Control. This test was performed on every individual new bottle of tissue culture media prior to use. 10 ml of sample media was taken aseptically and inoculated into each of 2 bottles of thioglycolate broth, one of which was incubated at



37°C, and the other at 33°C. After 7 days, if the broth remained clear, the medium was considered sterile and used throughout.

v. Tumour Cell Line - K562.

This cell line comes originally from the pleural effusion of a leukaemia patient in blast crisis. The K562 cell line was donated by Dr. D. Jones, Department of Pathology, University of Southampton Medical School. This in vitro tumour cell line was maintained as a stationary suspension culture in RPMI-1640 (with 10% NBCS). (Lozzio and Lozzio 1975).

vi. Raji Tumour Cell Line.

Raji cells, which are derived from a Burkitts' lymphoma, were cultured in RPMI 1640 (with 10% NBCS) as a stationary suspension (The Raji cells were obtained from Flow Laboratories, Irvine, Scotland). Cells used in the assay were harvested 72 hours after initiation of the culture.

vii. Maintenance of Tissue Culture.

Stationary suspension cultures. All these lines were subcultured regularly when confluent. Bottles were generally split in a ratio of 1 to 5 with fresh culture medium and the cells reincubated at 37°C.

viii. Viable Cell Counts.

Viable cell counts of tumour cell suspension were performed using Trypan Blue exclusion, where dead cells take up the dye within a few seconds and appear blue under the microscope, making them easily distinguishable from viable cells. One part cells (0.2 ml) to 1 part Trypan Blue (0.2 ml) to 8 parts (1.6 ml) of phosphate buffered

saline were mixed and placed under the coverslip of an improved Neubauer Counting Chamber (Hawksly Limited, Lancing, Sussex).

Magnification at 100-400 times was used to count the cells and cell suspensions of less than 90% viability were discarded.

Lymphocyte populations were also counted using the same technique.

ix. African Green Monkey Kidney Cell Line. Method of Subculture.

This cell line was obtained from Flow Laboratories, Irvine.

Confluent cell monolayers were washed in PBS and then 10 ml of trypsin/versene mixture (1:1 ratio) was layered onto the monolayer.

After gentle agitation the mixture was decanted leaving a film over the cell monolayer which was then incubated at 37°C for 1 hour. The cells were then washed off the glass culture bottle with Medium 199, counted and a cell suspension prepared and transferred to a glass culture bottle, incubated at 37°C which resulted in a confluent monolayer in 3 to 4 days.

x. Baby Hamster Kidney Cells BHK(21).

Cell cultures were obtained from Flow Laboratories, Irvine, Scotland. These were cultured as monolayers with trypsin into minimal essential medium at 37°C. Confluent monolayers were obtained in 3 to 4 days.

6. SEMLIKI FOREST VIRUS.

The Semliki Forest virus was a gift from Dr. M.D. Johnston, Wellcome Research Laboratories, Beckenham, Kent.

Baby hamster kidney cells (BHK21) were grown to confluent monolayers in a 20 oz glass bottle. One ml of 1/100 dilution of stock solution virus was inoculated onto the cell monolayer, allowed to adsorb for 1 hour at 37°C and

then the inoculum removed. Thirty ml of medium 199 containing 2% NBCC was added to the culture bottle which was incubated for 36 hours at 37°C. The supernatant containing the virus was then removed, clarified into aliquots and stored at -80°C prior to use.

## 7. MONOCLONAL ANTIBODIES.

Monoclonal antibodies (Becton Dickenson) were supplied by Labarotory Impex, Lion Road, Twickenham, London. The following monoclonal antibodies were used to identify the lymphocyte populations studied.

Leu 4 (pan T lymphocytes)

Leu 7 (NK lymphocyte)

## 8. EXPERIMENTAL METHODS.

### i. Blood Sampling.

- a. Peripheral venous blood. Five to 20 ml of peripheral venous blood was taken from the antecubital vein using a tourniquet and venepuncture with a 19G needle. 5 ml samples were placed into lithium heparin tubes and 20 ml samples placed in a sterile universal container with 0.2 ml of heparin added. These samples were taken before induction of anaesthesia and prior to surgery.
- b. Samples taken during operation. Blood (2-5ml) was taken from the aorta, this was assumed to be equivalent to the arterial supply to the tumour in terms of NK cell activity, using a 25G needle and 10 ml syringe "lubricated" with heparin. The largest vein draining the tumour was also sampled using an identical technique and also a vein draining macroscopically normal

adjacent tissue (stomach or colon).

In the control group of patients with benign disease samples were taken from aorta, a stomach vein and a colon vein.

Peripheral venous samples were also taken during surgery one hour after induction of anaesthesia and start of surgery.

ii. Transport of blood samples.

The blood samples were either sent for analysis to the Virology Department, Medical School, Sheffield on the same day or kept overnight at 4°C for analysis the following morning.

iii. Anaesthetic Techniques.

Inhalation anaesthetics. After intravenous induction with thiopentone, a balanced gas anaesthetic of nitrous oxide, oxygen and halothane was given with neuromuscular blocking drugs and artificial ventilation.

Infusion anaesthetics. A total intravenous anaesthetic technique using Etomidate/Fentanyl and neuromuscular blocking drugs with artificial ventilation of the lungs was used in some patients with benign and malignant disease. A two step schedule was used with etomidate 100 µg/kg/minute and fentanyl 1 µg/kg/minute given for ten minutes followed by a maintenance dose at a one tenth of this rate.

iv. Lymphocyte Separation.

Whole blood. Twenty to 40 ml of heparinised whole blood was layered in 5 ml volumes on top of 4 ml of LSM in a centrifuge tube. The samples were centrifuged at 400 g for 30 minutes at room temperature which resulted in the lymphocytes forming a distinct layer at the interface of the plasma and LSM. This layer of cells was drawn up into a Pasteur pipette taking minimal amounts of plasma

and LSM. The lymphocyte suspension was washed 3 times in RPMI (Boyum 1968) counted and resuspended in a known volume of RPMI.

v. 4 Hour  $^{51}\text{Cr}$  Release Cytotoxicity Assay.

a. Labelling of Tumour Target Cells.

Target cells were spun down and resuspended in 0.2 ml of RPMI and labelled with  $\text{Na}_2^{51}\text{CrO}$  (Radio Chemical Centre, Amersham, Buckinghamshire) by adding 100  $\mu\text{Ci}$ /0.1 ml of the isotope and incubating for 1 hour at  $37^\circ\text{C}$ . These cells were then washed 3 times, reincubated in the fresh medium for 1 hour, washed (x3) and a trypan blue count performed. The cells were then resuspended in RPMI at a concentration of  $10^5$  cells/ml.

b.  $^{51}\text{Cr}$ -release Assay for Separated Lymphocytes.

Effector cells were adjusted to the required cell concentrations to give effector:target ratios of between 50:1 and 6:1 (effector cells at  $5 \times 10^6$  cells/ml to  $6 \times 10^5$  cells/ml). 0.1 ml of effector cells and 0.1 ml of  $^{51}\text{Cr}$ -labelled target cells were added (in triplicate) to round bottomed microtest plates (Falcon Products). In those tests where the effect of plasma from the patients with malignant disease was being studied, 0.05 ml of effector cells and 0.05 ml of plasma were added to 0.1 ml of  $^{51}\text{Cr}$ -labelled target cells. The test plates were incubated at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$ /95% air atmosphere for 4 hours. The cells were sedimented by centrifugation at 200 g for 5 minutes and 0.1 ml of supernatant removed into separate wells. The plates were dried in an oven, sprayed with Opsite plastic spray and then individual wells counted on a Packard gamma spectrophotometer. The  $^{51}\text{Cr}$  release was directly proportional to target cell killing and was counted as follows:



$$\% \text{ } ^{51}\text{Cr release} = \frac{2 \times (\frac{1}{2}\text{SN})}{(\text{cells} + \frac{1}{2}\text{SN}) + (\frac{1}{2}\text{SN})} \times 100$$

where SN = supernatant.

Percentage cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{test release} - \text{spontaneous release}}{100 - \text{spontaneous release}} \times 100$$

c.  $^{51}\text{Cr}$ -release Assay for Whole Blood.

Whole blood was serially diluted at 1/2, 1/4 and 1/8 dilutions with RPMI (10% NBCS) and 0.1 ml volumes placed in test wells with 0.1 ml of  $^{51}\text{Cr}$  labelled target cells (Rees and Platts 1983). The percent  $^{51}\text{Cr}$  release and percentage cytotoxicity was calculated in the identical manner for the assay for separated lymphocytes. The cytotoxic capacity of whole blood to K562 targets correlates well with the cytotoxicity of separated PBL's (Rees and Platts 1983).

vi. Preparation and Storage of Plasma Samples.

Whole blood was centrifuged at 400 g for 20 minutes, the plasma collected and then further centrifuged at 400 g, for 30 minutes. 1 ml aliquots were taken into sterile glass tubes which were stored at  $-80^{\circ}\text{C}$  prior to study.

vii. Addition of Plasma Samples to Control PBL's.

PBL's were separated from the peripheral venous blood of normal controls (Laboratory volunteers) as described previously. Fifty microlitres of plasma from the peripheral venous blood, or tumour draining venous blood of the tumour patients were added to 50  $\mu\text{l}$  control lymphocytes at concentrations of  $1 \times 10^6$  and  $5 \times 10^5$  cells per ml and pre incubated for 1 hour at  $37^{\circ}\text{C}$  in a humidified 95% air 5%  $\text{CO}_2$  atmosphere prior to cytotoxicity assay. In addition 50  $\mu\text{l}$  of plasma from the peripheral venous blood, stomach and colon



draining venous blood of patients with benign disease were added to 50  $\mu$ l of control lymphocytes at concentrations of  $1 \times 10^6$  and  $5 \times 10^5$  cells per ml and preincubated as above prior to cytotoxicity assay.

viii. Heat Treatment of Plasma.

Plasma samples were immersed in a water bath at  $56^{\circ}\text{C}$  for 40 minutes to inactivate complement and will be referred to as heat treated plasma (HTP). Fifty microlitres of HTP was then added to 50  $\mu$ l of control lymphocytes at concentrations of  $1 \times 10^6$  and  $5 \times 10^5$  cells per ml, the cells preincubated for 1 hour as before prior to cytotoxicity assay.

ix. Monoclonal Antibody Labelling of Lymphocytes and Microscopy.

A lymphocyte suspension was taken from the whole blood sample and washed 3 times in serum free medium (RPMI) prior to addition of monoclonal antibody. One hundred microlitres of the optimum dilution of the appropriate monoclonal antibody dilution was added to  $1 \times 10^6$  lymphocytes for 20 minutes at room temperature. The cells were washed 3 times in PBS and then 100  $\mu$ l of the optimum dilution of fluorescein conjugated (goat antimouse IgG) added at the appropriate dilution and incubated for a further 20 minutes at room temperature. The cells were washed 3 times in PBS and re-suspended in a small volume of saline, samples were kept in ice prior to examination by fluorescent microscopy on an ultraviolet microscope (Leitz) for surface fluorescence using water immersion at a magnification of  $\times 500$ . A total of between 150 and 200 cells were assessed for monoclonal antibody staining in each sample.

x. Raji Cell Assay for Detecting Immune Complexes.

This was based on the method of Theofilopoulos et al 1976. Test and control sera (25  $\mu$ l) which had previously been incubated for 30 minutes at 37°C with an equal volume of a 1 in 2 dilution of fresh pooled normal human serum (as a complement source) were added in triplicate to  $2 \times 10^6$  Raji cells in 70 x 12 mm polystyrene tubes.

A reference standard curve was prepared in parallel by heat aggregating (37°C for 30 minutes) Cohn fraction II, and adding aliquots to the Raji cells to give a final concentration range of 1.5  $\mu$ l to 400  $\mu$ g/ml. Only normal control tubes that (a) were free of both aggregate and serum, and (b) contained only normal human serum were included in the assay. After incubating for 30 minutes at 37°C, the cells were washed 3 times and then incubated for 45 minutes at 4°C with 80  $\mu$ l of  $^{125}$ I-staphylococcal protein A which had been iodinated with Iodogen, and used at an optimum dilution at which saturated 1 gG bound to Raji cells. The cells were finally washed 3 times and the radioactivity bound to the pellet counted and, by reference to the standard curve the results expressed as  $\mu$ g/ml equivalent of the aggregated Cohn fraction II.

xi. Total White Blood Cell Counts Morphological Studies of White Blood Cells.

Conventional microscopy using a Neubauer counting chamber and Giemsa staining was performed to determine the total white blood cell count of blood samples. In addition morphological studies of the white blood cells were carried out to characterise the nature of the white cell population in terms of neutrophils, monocytes, large and small lymphocytes.

xii. Interferon Assay.

Plasma interferon levels were measured by the inhibition of cytopathic effect method (Johnston 1981) as determined by uptake of a vital dye. Confluent monolayers of the African Green Monkey kidney cell line, V3, in flat bottomed wells of microtest plates, were incubated in the presence of serial dilutions of patients' plasma for 16 hours prior to addition of the challenge virus, Semliki forest virus. The diluent was medium 199 containing 2% foetal calf serum and all incubations were carried out at 37°C in a humidified 5% CO<sub>2</sub> in air atmosphere. Cells surviving a 48 hour challenge period were stained with 0.025% (w/v) crystal violet in 5% (V/v) formaldehyde. After 90 minutes at 37°C, the test plates were washed with distilled water and the 50% protection end points determined by inspection. All interferon titres are given in log<sub>10</sub> IU/ml. A laboratory reference human interferon standard, which had been calibrated against the International Reference Preparation 69/19, was included as a positive control.

xiii. In Vitro Incubation of PBL's with Exogenous Interferon.

Lymphocytes were separated from heparinized peripheral venous blood by Ficoll-Hypaque density gradient centrifugation, washed in RPMI 3 times, counted and resuspended at a concentration of 10<sup>6</sup> cells per ml. Half of the cells were used in a 4 hour cytotoxicity assay similar to the assay described for whole blood, at effector to target ratios of 50, 25 and 12.5 to 1. The remaining cells were incubated for 18 hours in a humidified CO<sub>2</sub> atmosphere in the presence of 100 IU of human leucocyte interferon per 10<sup>6</sup> cells. These cells were then washed 3 times in RPMI, counted and resuspended at 10<sup>6</sup> cells/ml prior to cytotoxicity assay.

CHAPTER 4.

## CHAPTER 4.

### Experimental results relating to the tumour effect on natural killer lymphocytes.

- 4.1 Introduction
- 4.2 Cytotoxicity of peripheral venous blood of patients with cancer and benign disease against K562 target cells.
- 4.3 Natural cytotoxicity of arterial blood supplying the tumour and venous blood draining the tumour during surgical operation.
- 4.4 Natural cytotoxicity of arterial blood supplying the gut and of venous blood draining the stomach and colon in benign patients during surgical procedure.
- 4.5 The effect of autologous plasma from benign controls on natural cytotoxicity of peripheral blood lymphocytes.
- 4.6 The effect of plasma from patients with cancer on K562 target cells.
- 4.7 The influence of plasma from cancer patients on natural cytotoxicity to K562 targets by lymphocytes from benign controls.
- 4.8 The effect of plasma from patients with benign abdominal disease on natural cytotoxicity to K562 targets by lymphocytes from normal controls.
- 4.9 The effect of heat treatment of plasma from patients with benign disease on natural cytotoxicity of PBL's from normal controls.
- 4.10 The effect of heat treatment of plasma from patients with malignant disease on natural cytotoxicity of PBL's from normal controls.



- 4.11 Assay for immune complexes in peripheral venous and tumour draining venous blood.
- 4.12 Studies of white blood cell counts by morphology across the tumour circulation.
  - a. total white blood cell counts.
  - b. total lymphocyte counts.
  - c. polymorphonuclear leucocyte counts.
- 4.13 Studies using the monoclonal antibody Leu 7 on lymphocytes transversing the tumour circulation.
- 4.14 Discussion.



#### 4.1 Introduction.

In view of the findings of several workers that natural killer cell activity was reduced in patients with cancer (Pross and Baines 1976, Kadish et al 1981) and particularly that natural killer cell activity in the host was related to tumour burden (Pross and Baines 1976, Horowitz et al 1983), the experiments described in this chapter were undertaken to determine the influence of the tumour on NK activity of the host and also to study the effect of passage through the tumour circulation on the capacity of host natural killer lymphocytes to kill tumour target cells (K562). Further studies were undertaken to determine changes in lymphocyte subpopulations, particularly the natural killer lymphocyte fraction, in the blood across the tumour circulation. In addition the plasma taken from the peripheral venous, aortic, tumour venous, stomach and colon venous blood of patients with cancer and similar blood samples including tumour venous blood from patients with benign abdominal disease was added to PBL's from healthy controls to determine their influence on the natural cytotoxicity of control PBL's to tumour target cells. This was undertaken to determine whether factors present in the efferent venous blood from tumours were inhibitory to exogenous NK cells taken from normal individuals.

#### 4.2 Cytotoxicity of peripheral venous blood of patients with cancer and benign disease against K562 target cells.

Cytotoxicity of peripheral venous blood to K562 target cells was determined by the whole blood assay described in the materials and methods section. Nineteen patients with benign abdominal disease and 27 patients with gastrointestinal cancer were studied. Of the 27 patients

with cancer, 18 patients had localised primary tumours confined to the organ of origin, i.e. either stomach or colon, and 8 patients had disseminated disease with distant metastases. Blood samples were taken from each patient prior to anaesthesia and surgery and the blood tested at dilutions of 1/2, 1/4 and 1/8 for natural cytotoxicity. Patient details and results expressed as percentage cytotoxicity are shown in Table I.

The groups were not matched for age and sex. The patients with malignant disease were older than those with benign disease ( $p < 0.01$  by Students "t" test), however this was not thought to influence NK status significantly since this is known not to be age related in the adult human (Takasugi et al 1973). In addition, regression analysis of percentage cytotoxicity of peripheral venous blood versus age in years of 18 patients with benign disease ( $r = 0.09$ ) and 27 patients with malignant disease ( $r = -0.012$ ) failed to show any statistically significant correlation of NK status with age. (Fig.1.)

There was no significant difference in the mean cytotoxicity of the patients with localised primary tumours compared to the patients with benign abdominal disease at either dilution studied (Table I). There was however, a significant reduction of the mean cytotoxicity at 1/2 and 1/4 dilutions ( $p < 0.005$  by Students "t" test) for patients with disseminated malignant disease when compared to the benign controls. This finding implies that NK lymphocyte mediated cytotoxicity of peripheral venous blood against K562 target cells is directly proportional to tumour burden rather than the mere existence of the primary tumour itself.

TABLE I

## Patient details

## i) Benign disease

n = 19 7 M 12 F

Mean age  $\pm$  SEM 53.2  $\pm$  3.7 years

## ii) Local malignant disease

n = 19 12 M 7 F

Mean age  $\pm$  SEM 66.6  $\pm$  3.0 years

## iii) Disseminated malignant disease

n = 8 8 M 0 F

Mean age  $\pm$  SEM 66.7  $\pm$  2.45 yearsMEAN PERCENTAGE CYTOTOXICITY  $\pm$  SEM OF PERIPHERAL  
VENOUS BLOOD TO K562 TARGETS

Dilution of whole blood	Benign	Local malignant disease	Disseminated malignant disease
$1/2$	16.3 $\pm$ 2.7	11.2 $\pm$ 1.5	7.0 $\pm$ 1.3*
$1/4$	9.3 $\pm$ 1.15	7.5 $\pm$ 1.38	4.1 $\pm$ 1.11*
$1/8$	5.5 $\pm$ 0.75	4.15 $\pm$ 0.89	2.9 $\pm$ 0.76

\*  $p < 0.005$ 

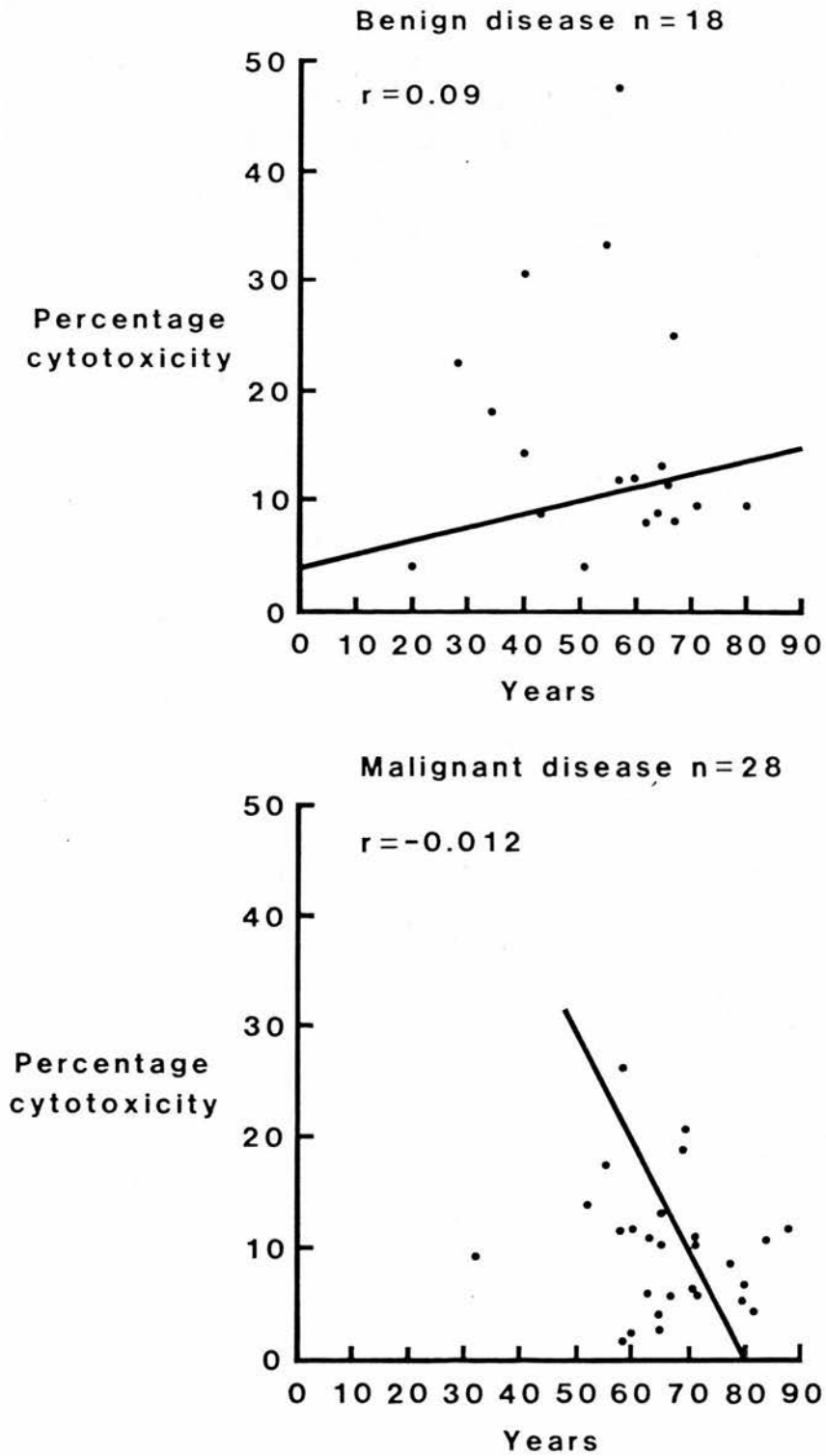
4 hr. chromium release assay of peripheral venous whole blood against K562 targets.

 $1/2$  dilution with RPMI.

Statistical analysis by Student's "t" test.

FIGURE 1

THE FAILURE OF CORRELATION BETWEEN CYTOTOXICITY OF  
PERIPHERAL VENOUS BLOOD TO K562 TARGET CELLS AND AGE IN PATIENTS  
WITH BENIGN AND MALIGNANT DISEASE



4 hr. chromium release assay against K562 targets using peripheral venous whole blood at  $1/2$  dilution.

#### 4.3 Natural cytotoxicity of arterial blood supplying the tumour during surgical operation.

In 22 patients with histologically proven malignant disease of the gastrointestinal tract, it was possible to sample aortic blood, representing the arterial blood supplying the tumour, and venous blood directly draining the tumour to assess natural cytotoxicity against K562 targets. In 16 of these patients the tumour was an adenocarcinoma of the large bowel, 4 patients had an adenocarcinoma of the stomach and in the remaining 2 patients the tumour was a gastric lymphoma. The results of the whole blood assay for natural killer cell activity in the aortic blood and tumour venous blood against K562 targets are shown in Table II a (1/2 dilution of the whole blood with RPMI) and Table II b (1/4 dilution with RPMI). Since each test is performed in triplicate the results are expressed as mean percentage cytotoxicity  $\pm$  one standard deviation which enables statistical analysis by the paired "t" test of the cytotoxicity of whole blood entering the tumour (aortic blood) compared to the cytotoxicity in the tumour venous draining blood. At the 1/2 dilution studied (Table IIa), 4 patients (JC, FW, WD and BR) had statistically significant ( $p < 0.05$ – $p < 0.01$ ) depression of cytotoxicity of blood after passage through the tumour circulation. In 3 of these patients (JC, FW, WD) the tumour was an adenocarcinoma of the colon and the remaining patient (BR) had an adenocarcinoma of the stomach. Three patients (JB, PW and WN) had significant elevation ( $p < 0.05$ – $p < 0.001$ ) of cytotoxicity in the tumour draining venous blood compared to the aortic blood entering the tumour circulation. The tumours in these 3 patients were an adenocarcinoma of the colon (JB), lymphoma of stomach (PW) and an adenocarcinoma of stomach (WN) respectively.

TABLE IIA

PERCENTAGE CYTOTOXICITY  $\pm$  SD OF WHOLE BLOOD TO K562  
TARGETS ENTERING AND LEAVING THE TUMOUR CIRCULATION

$\frac{1}{2}$  DILUTION OF WHOLE BLOOD

Patient	Tumour	Aortic	Tumour venous	P value
JC	Colon	23.5 $\pm$ 5.4	6.8 $\pm$ 2.2	< 0.01 $\downarrow$
RR	Colon	14.3 $\pm$ 4.3	16.4 $\pm$ 3.4	NS
FO	Colon	13.0 $\pm$ 1.0	10.5 $\pm$ 2.6	NS
GW	Colon	7.0 $\pm$ 1.5	6.5 $\pm$ 0.6	NS
DG	Colon	14.9 $\pm$ 3.1	11.4 $\pm$ 0.36	NS
JB	Colon	14.6 $\pm$ 0.7	20.3 $\pm$ 3.3	< 0.001 $\uparrow$
WW	Colon	15.8 $\pm$ 1.5	23.3 $\pm$ 7.1	NS
FW	Colon	43.1 $\pm$ 14.4	18.4 $\pm$ 1.7	< 0.05 $\downarrow$
NF	Colon	10.9 $\pm$ 1.5	10.7 $\pm$ 3.4	NS
GH	Colon	7.6 $\pm$ 3.8	3.4 $\pm$ 0.5	NS
WD	Colon	18.6 $\pm$ 1.4	11.0 $\pm$ 2.9	< 0.01 $\downarrow$
DP	Colon	29.2 $\pm$ 8.6	13.9 $\pm$ 3.4	NS
MS	Colon	5.2 $\pm$ 2.4	3.9 $\pm$ 2.6	NS
NW	Colon	16.9 $\pm$ 2.5	15.7 $\pm$ 4.4	NS
AH	Colon	3.4 $\pm$ 1.9	5.0 $\pm$ 3.0	NS
AP	Colon	3.6 $\pm$ 1.8	2.5 $\pm$ 3.1	NS
PW	Stomach	29.5 $\pm$ 3.7	35.0 $\pm$ 2.6	< 0.01 $\uparrow$
JC	Stomach	25.0 $\pm$ 1.7	18.8 $\pm$ 3.3	NS
WN	Stomach	7.6 $\pm$ 1.3	18.8 $\pm$ 5.4	< 0.05 $\uparrow$
FP	Stomach	2.9 $\pm$ 2.2	10.5 $\pm$ 2.6	NS
BR	Stomach	37.7 $\pm$ 6.2	26.7 $\pm$ 5.4	< 0.01 $\downarrow$
HW	Stomach	4.6 $\pm$ 3.0	7.5 $\pm$ 2.5	NS

4 hr. chromium release cytotoxicity assay for whole blood against K562 targets.

Statistical analysis by paired "t" test.



TABLE IIB

PERCENTAGE CYTOTOXICITY  $\pm$  SD OF WHOLE BLOOD TO K562  
TARGETS ENTERING AND LEAVING THE TUMOUR CIRCULATION

1/4 DILUTION OF WHOLE BLOOD

Pt.	Tumour	Aortic	Tumour venous	p value
JC	Colon	19.2 $\pm$ 2.7	4.5 $\pm$ 1.4	<0.001 $\downarrow$
RR	Colon	9.7 $\pm$ 0.5	13.5 $\pm$ 0.7	0.01 $\uparrow$
FO	Colon	6.5 $\pm$ 1.5	5.5 $\pm$ 1.5	NS
GW	Colon	3.5 $\pm$ 1.4	4.9 $\pm$ 0.8	NS
DG	Colon	11.4 $\pm$ 3.9	6.5 $\pm$ 2.2	<0.05 $\downarrow$
JB	Colon	8.5 $\pm$ 0.8	16.9 $\pm$ 2.2	<0.01 $\uparrow$
WW	Colon	8.2 $\pm$ 3.3	11.8 $\pm$ 4.1	NS
FW	Colon	21.4 $\pm$ 3.0	16.7 $\pm$ 9.8	NS
NF	Colon	18.9 $\pm$ 1.5	21.3 $\pm$ 1.4	NS
GM	Colon	7.7 $\pm$ 6.0	2.7 $\pm$ 1.6	NS
WD	Colon	19.1 $\pm$ 2.7	7.8 $\pm$ 1.8	<0.02 $\downarrow$
DP	Colon	15.2 $\pm$ 4.2	0.5 $\pm$ 0.5	<0.01 $\downarrow$
MS	Colon	0.9 $\pm$ 0.8	2.0 $\pm$ 1.9	NS
NW	Colon	11.8 $\pm$ 2.1	12.2 $\pm$ 2.2	NS
AM	Colon	0.9 $\pm$ 0.9	3.6 $\pm$ 3.1	NS
AP	Colon	0.0 $\pm$ 0.3	2.6 $\pm$ 0.8	NS
PW	Stomach	23.2 $\pm$ 5.3	30.0 $\pm$ 4.3	NS
JC	Stomach	10.5 $\pm$ 10.0	3.8 $\pm$ 1.6	NS
WN	Stomach	6.5 $\pm$ 1.0	17.7 $\pm$ 3.1	<0.01 $\uparrow$
FP	Stomach	1.5 $\pm$ 0.7	3.7 $\pm$ 2.7	NS
BR	Stomach	33.0 $\pm$ 1.1	26.9 $\pm$ 3.1	<0.05 $\downarrow$
HW	Stomach	3.5 $\pm$ 1.6	5.3 $\pm$ 1.6	NS

4 hr. chromium release cytotoxicity for whole blood against K562 targets.

Statistical analysis by paired "t" test.

In the remaining 15 patients (12 colon tumours, 3 stomach tumours) there was no significant change in cytotoxicity of whole blood after passage through the tumour circulation.

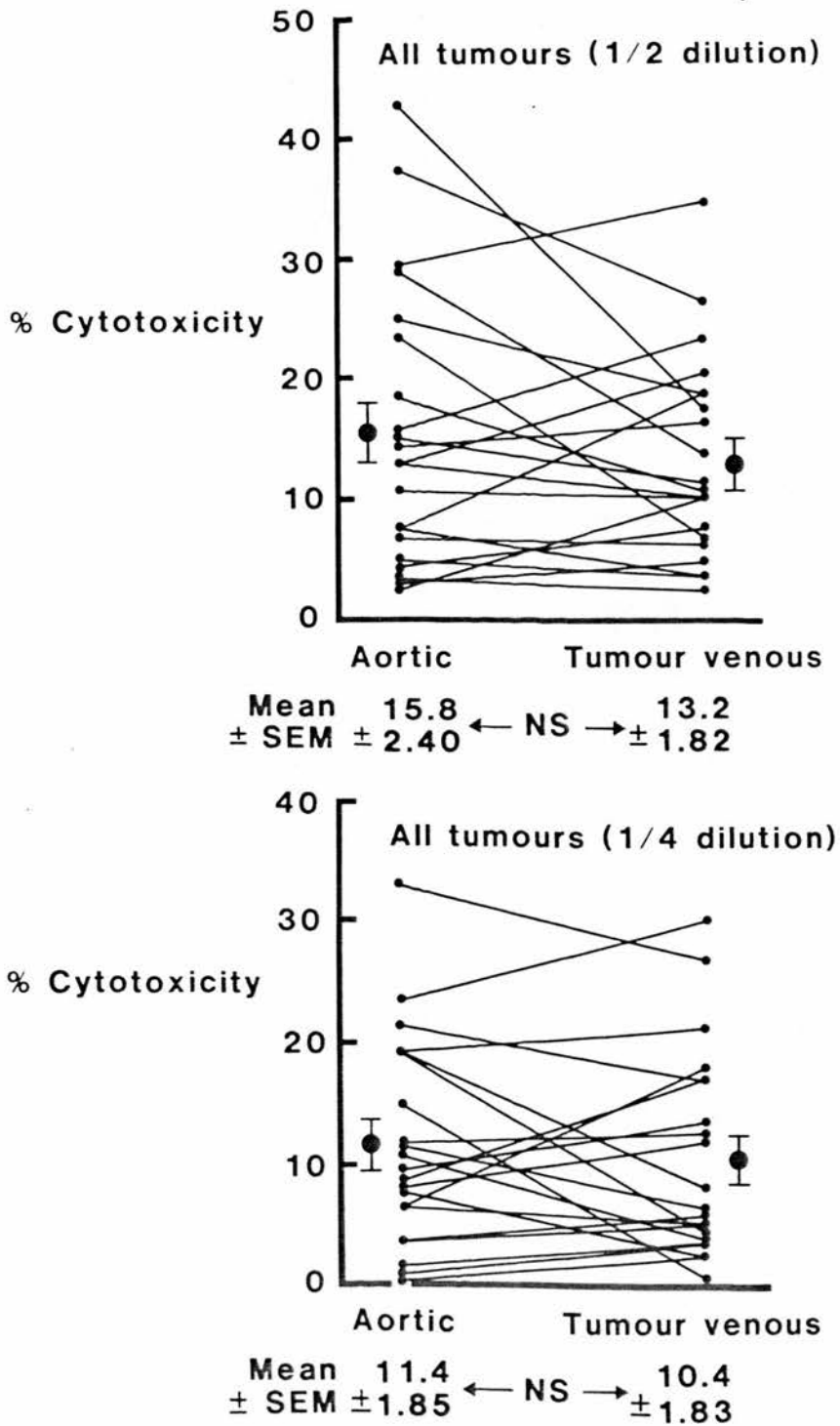
At the 1/4 dilution studied, (Table IIb) 5 patients (JC, DG, WD, DP and BR) showed significant depression ( $p < 0.05$ – $p < 0.001$ ) of cytotoxicity of whole blood in the tumour draining venous blood compared to the aortic blood supplying the tumour. Four of these patients (JC, DG, WD and DP) had an adenocarcinoma of the large bowel and the remaining patient (BR) an adenocarcinoma of the stomach. In 3 patients (RB, JB, and WN) there was significant elevation ( $p < 0.05$ – $p < 0.01$ ) of cytotoxicity in the tumour draining venous blood compared to the aortic blood supplying the tumour. The tumours in these patients were an adenocarcinoma of the colon in 2 patients (RB, JB) and an adenocarcinoma of the stomach in the remaining patient (WN). At this dilution there were 14 patients (10 colon tumours, 4 stomach tumours) in whom there was a statistically insignificant change in cytotoxicity in the aortic blood compared to the tumour draining venous blood.

Analysis of the mean cytotoxicity by the paired "t" test for the aortic and tumour venous blood at the 1/2 and 1/4 dilutions for all tumours is shown in Figure 2. The mean percentage cytotoxicity  $\pm$  SEM for the aortic blood at the 1/2 dilution was  $15.8 \pm 2.4\%$  which did not differ significantly from the mean percentage cytotoxicity  $\pm$  SEM for the tumour draining venous blood at  $13.2 \pm 1.82\%$ . Similarly at the 1/4 dilution studied, there was no significant difference in the mean percentage cytotoxicity  $\pm$  SEM of the aortic blood at  $11.4 \pm 1.85\%$  compared to that of the tumour draining venous blood at  $10.4 \pm 1.83\%$ .

These data suggest that in the majority of patients with gastrointestinal

FIGURE 2

CYTOTOXICITY OF WHOLE BLOOD TO K562 TARGETS OF AORTIC BLOOD  
AND TUMOUR VENOUS BLOOD



4 hr. chromium release assay.

Results are the mean of 3 separate tests for each patient.

Statistical analysis by the paired "t" test.

tumours, there is no significant depression or elevation of natural cytotoxicity of whole blood to K562 tumour target cells after passage through the tumour circulation. There is however a degree of individual patient variation with, in some patients, depression of natural cytotoxicity of whole blood after passage through the tumour whilst in other patients there appears to be an enhancement of natural cytotoxicity. In these patients, there may be factors active in the tumour circulation which could result in either depression or enhancement of host natural cytotoxicity.

#### 4.4 Natural cytotoxicity of arterial blood supplying the gut and of venous blood draining the stomach and colon in benign patients during surgical operation.

In order to ascertain whether there existed a gradient of natural killer cell activity across the stomach and colon of patients without gastrointestinal tumours, blood samples were taken during operation on 10 patients with benign upper abdominal conditions. Five of these patients were undergoing cholecystectomy for gallstones, 3 patients had surgery for duodenal ulcer disease and the remaining 2 patients partial gastrectomy for benign gastric ulcer. Blood was taken from the aorta and also veins draining the stomach and colon, diluted with RPMI to 1/2 and 1/4 dilution and then estimation of natural cytotoxicity against K562 target cells was performed. The results are expressed as percentage cytotoxicity  $\pm$  SD and shown in Table III.

There was some variation between patients in the natural cytotoxicity of arterial blood supplying the gut and venous blood draining the stomach and colon.

At the 1/2 dilution studied, in 2 patients (GK, EB) there was significant ( $p < 0.01, p < 0.001$ ) depression of natural cytotoxicity to K562 targets in the stomach venous blood compared to the aortic blood supplying the gut and in one patient (GB), natural cytotoxicity was significantly ( $p < 0.01$ ) elevated in the stomach venous blood. Only one patient (MG) showed any significant change in natural cytotoxicity of the colon venous blood with a significant depression ( $p < 0.01$ ) of cytotoxicity when compared to the aortic blood. In the remaining patients there was no significant change of natural cytotoxicity of blood draining the stomach and colon when compared to the aortic blood.

At the 1/4 dilution studied, in one patient (NT) there was significant depression ( $p < 0.01$ ) of stomach draining venous blood cytotoxicity compared to the cytotoxicity of the aortic blood supplying the gut. One patient (GK) showed depression ( $p < 0.01$ ) of cytotoxicity in the blood draining the colon compared to aortic blood and a further patient (PB) showed significant ( $p < 0.01$ ) elevation of natural cytotoxicity of blood draining the colon. All of the remaining patients failed to show any significant change in natural cytotoxicity of blood draining the stomach and colon circulations when compared to the natural cytotoxicity of aortic blood supplying the gut.

In Table IV, the mean percentage cytotoxicities to K562 of the 3 tests performed on the aortic, stomach venous and colon venous blood of each patient at 1/2 and 1/4 dilutions with RPMI are shown. There is no statistically significant difference between the mean  $\pm$  SEM aortic cytotoxicity at  $25.0 \pm 5.1\%$  and that of the stomach draining venous blood at  $25.1 \pm 4.9\%$  and the colon draining venous blood at  $21.7 \pm 4.5\%$  at the 1/2 dilution studied. Similarly at the 1/4 dilution, the



TABLE III

PERCENTAGE CYTOTOXICITY  $\pm$  SD OF WHOLE BLOOD TO K562  
TARGETS IN AORTIC BLOOD, STOMACH AND COLON VENOUS BLOOD  
OF PATIENTS WITH BENIGN ABDOMINAL DISEASE

$\frac{1}{2}$ DILUTION						
Patient	Diagnosis	Aortic	Stomach venous	P	Colon venous	P
MB	Gallstones	$11.1 \pm 1.2$	$3.7 \pm 2.1$	NS	$9.4 \pm 1.8$	NS
NT	Gallstones	$59.3 \pm 2.3$	$52.5 \pm 1.6$	NS	$27.9 \pm 2.0$	NS
GK	D U	$10.9 \pm 1.1$	$3.6 \pm 1.5$	$< 0.01 \downarrow$	$9.3 \pm 2.7$	NS
GB	D U	$9.8 \pm 1.3$	$20.7 \pm 3.4$	$< 0.01 \uparrow$	$11.8 \pm 1.4$	NS
EB	Gallstones	$34.8 \pm 3.9$	$20.7 \pm 2.5$	$< 0.001 \downarrow$	NT	
MG	Gallstones	$20.5 \pm 2.1$	$17.6 \pm 0.2$	NS	$10.4 \pm 0.9$	$< 0.01 \downarrow$
WW	D U	$32.0 \pm 3.7$	$26.4 \pm 0.9$	NS	$36.7 \pm 5.9$	NS
PB	Gallstones	$33.0 \pm 1.2$	$44.0 \pm 7.7$	NS	$36.9 \pm 7.2$	NS
MT	G U	$10.5 \pm 2.2$	$14.5 \pm 1.2$	NS	$13.2 \pm 1.2$	NS
FA	G U	$8.0 \pm 2.1$	$11.0 \pm 8.5$	NS	$8.2 \pm 1.1$	NS

$\frac{1}{4}$ DILUTION						
Patient	Diagnosis	Aortic	Stomach venous		Colon venous	
MB	Gallstones	$9.3 \pm 2.3$	$12.0 \pm 7.5$	NS	$8.5 \pm 2.0$	NS
NT	Gallstones	$9.2 \pm 2.0$	$0.1 \pm 0.05$	$< 0.01 \downarrow$	$8.4 \pm 2.5$	NS
GK	D U	$25.8 \pm 2.2$	$33.9 \pm 5.2$	NS	$17.2 \pm 3.6$	$< 0.01 \downarrow$
GB	D U	$8.2 \pm 2.9$	$11.7 \pm 2.7$	NS	$6.8 \pm 1.5$	NS
EB	Gallstones	$22.4 \pm 3.5$	$15.9 \pm 7.5$	NS	NT	
MG	Gallstones	$12.7 \pm 4.6$	$8.5 \pm 1.4$	NS	$8.8 \pm 0.6$	NS
WW	D U	$12.4 \pm 2.7$	$19.2 \pm 2.0$	NS	$29.6 \pm 0.8$	NS
PB	Gallstones	$20.3 \pm 2.0$	$25.5 \pm 8.4$	NS	$26.7 \pm 0.9$	$< 0.01 \uparrow$
MT	G U	$7.4 \pm 3.5$	$9.4 \pm 1.8$	NS	$9.3 \pm 1.9$	NS
FA	G U	$11.5 \pm 1.3$	$9.0 \pm 3.6$	NS	$4.2 \pm 3.6$	NS

4 hr. chromium release cytotoxicity assay.

Statistical analysis by paired "t" test.

NS = not significant      NT = not tested



TABLE IV

PERCENTAGE CYTOTOXICITY OF AORTIC BLOOD,  
STOMACH AND COLON DRAINING VENOUS BLOOD  
TO K562 TARGETS IN PATIENTS WITH BENIGN  
ABDOMINAL DISEASE

	$\frac{1}{2}$ DILUTION		
	A	SV	CV
	11.1	3.7	9.4
	59.3	52.5	27.9
	10.9	3.6	9.4
	9.8	20.7	11.8
	34.8	20.7	NT
	20.5	17.6	10.4
	32.0	26.4	36.7
	33.0	44.0	36.9
	10.5	14.5	13.2
	8.0	11.0	8.2
Mean	25.0	25.1	21.7
$\pm$ S.E.M.	$\pm 5.1$	$\pm 4.9$	$\pm 4.5$
	← NS →		
P value	← NS →		

	$\frac{1}{4}$ DILUTION		
	A	SV	CV
	9.3	0	8.5
	9.2	0.1	8.4
	25.8	33.9	17.2
	8.2	11.7	6.8
	22.4	15.9	NT
	12.7	8.5	8.8
	12.4	19.2	29.6
	20.3	25.5	26.7
	7.4	9.4	9.3
	11.5	9.0	4.2
Mean	13.9	13.2	11.3
$\pm$ S.E.M.	$\pm 2.0$	$\pm 3.3$	$\pm 3.2$
	← NS →		
P value	← NS →		

4 hr. chromium release assay of whole blood against K562 targets.

A = aortic      SV = Stomach venous      CV = Colon venous

Individual patients percentage cytotoxicity expressed as a mean of 3 tests.

Statistical analysis by paired "t" test.

differences in the mean  $\pm$  SEM aortic cytotoxicity at  $13.9 \pm 2.0\%$  and the stomach venous at  $13.2 \pm 3.3\%$  and the colon venous at  $11.1 \pm 3.2\%$  are not statistically significant.

The results of these experiments suggest that there is no consistent effect with either marked elevation or depression of natural killer lymphocyte activity after passage through the circulation of stomach and colon in patients with non malignant disease.

#### 4.5 The effect of autologous plasma from benign controls on natural cytotoxicity of peripheral blood lymphocytes.

These experiments were undertaken to determine whether autologous plasma from laboratory staff whose lymphocytes were used as benign controls in the experiments described later in this chapter, had either a stimulating or inhibitory effect on the cytotoxicity of these lymphocytes. Lymphocytes were separated from the peripheral venous blood of 8 control subjects as described in the materials and methods section. A lymphocyte suspension in RPMI was prepared at a concentration of  $1 \times 10^6$  cells/ml and  $0.5 \times 10^6$  cells/ml to give effector to target ratios of 50 and 25 to 1 respectively.

Fifty microlitres of the lymphocyte suspension was incubated with 50 $\mu$ l of autologous plasma for 1 hour at 37°C prior to use in a cytotoxicity assay against K562 target cells. The results are shown in Table V.

There was no statistically significant difference between the mean  $\pm$  SEM cytotoxicity of the lymphocytes incubated in RPMI ( $38.6 \pm 3.7\%$  and  $23.7 \pm 3.0\%$ ) compared to those incubated with autologous plasma ( $36.3 \pm 4.9\%$  and  $20.3 \pm 4.2\%$ ) at effector to target ratios of 50:1 and 25:1 respectively. This experiment showed that incubation of the lymphocytes with autologous plasma did not enhance or depress cytotoxicity to K562 target cells.

TABLE V

THE EFFECT OF THE ADDITION OF AUTOLOGOUS PLASMA TO  
PBL'S ON PERCENTAGE CYTOTOXICITY TO K562 TARGET CELLS

n = 8

Effector : target ratio 50 : 1

	PBL'S + RPMI		PBL'S + AUTOLOGOUS PLASMA
	35.7		51.0
	38.5		19.7
	44.1		36.6
	50.1		49.8
	40.7		40.5
	28.3		31.1
	55.7		48.8
	24.3		13.4
Mean	39.6	NS	36.3
±S.E.M.	±3.7		±4.9

Effector : target ratio 25 : 1

	PBL's + RPMI		PBL'S + AUTOLOGOUS PLASMA
	31.7		38.7
	16.4		12.1
	34.5		21.3
	13.8		10.9
	31.8		31.1
	20.2		20.8
	27.3		20.6
	14.4		8.2
Mean	23.7	NS	20.3
±S.E.M.	±3.0		±4.2

4 hr. chromium release cytotoxicity release assay

Statistical analysis by the paired "t" test.

NS = not significant

#### 4.6 The effect of plasma from patients with cancer on K562 target cells.

In these experiments, the effect of incubation of K562 tumour target cells with plasma from the peripheral venous blood, aortic and tumour draining venous blood from 11 patients with gastrointestinal cancer was studied to establish whether the plasma alone from patients with cancer had any cytotoxic effect against K562 tumour target cells. One hundred microlitres of the plasma from either the peripheral venous blood, aortic blood or venous blood draining the tumour was added to 100  $\mu\text{L}$  of K562 target cells at a concentration of  $1 \times 10^5$  cells/ml which had previously been labelled with  $^{51}\text{Cr}$ . The test plates were incubated for 4 hours at  $37^\circ\text{C}$  prior to estimation of radioactivity of the supernatant. The results are expressed as percentage  $^{51}\text{Cr}$  release (see materials and methods section) and are shown in Table VI.

The percentage  $^{51}\text{Cr}$  release following incubation of the K562 tumour cells with plasma from the cancer patients was not significantly different from the spontaneous release of the K562 cells incubated in RPMI (i.e. target control). This indicates that the plasma from the cancer patients and particularly the plasma from the venous blood draining the tumour does not have any significant cytotoxic effect on K562 tumour cells.

#### 4.7 The influence of plasma from cancer patients on natural cytotoxicity to K562 targets by lymphocytes from benign controls.

Blood samples were taken during surgery from patients with gastrointestinal tumours as follows:

- i. Peripheral venous blood.
- ii. Aortic blood.
- iii. Tumour draining venous blood.

TABLE VI

THE EFFECT OF PLASMA ALONE FROM CANCER PATIENTS ON  
K562 TARGET CELLS ON RELEASE OF  $^{51}\text{Cr}$ . COMPARED TO  
SPONTANEOUS RELEASE FROM TARGET CELLS

Patient	Target control (i.e. K562 + media)	K562 + PV plasma	K562 + A plasma	K562 + TV plasma
JC	14.9	13.4	15.6	14.9
PW	5.9	9.8	7.6	10.7
RR	13.6	10.0	9.0	13.2
GW	3.5	5.1	7.1	6.0
DG	13.4	14.9	12.3	14.1
JB	14.4	14.5	13.4	15.0
FW	13.8	12.8	13.8	12.4
BR	9.1	10.0	12.3	13.9
MS	6.3	7.4	10.1	7.4
AM	9.3	9.6	8.7	11.7
HW	15.2	15.1	14.3	13.8
Mean	10.8	11.1	11.2	12.1
$\pm$ S.E.M.	$\pm 1.26$	$\pm 0.98$	$\pm 0.87$	$\pm 0.9$

PV = peripheral venous      A = aortic      TV = tumour venous

Spontaneous release of  $^{51}\text{Cr}$  after 4 hrs. Individual results are the mean of 3 tests.

The differences between the mean release of  $^{51}\text{Cr}$  are not statistically significant for K562 incubated in PV, A, TV plasma compared to control.

- iv. Blood from a vein draining macroscopically normal tissue 15 cm or more distant to the tumour.

Plasma was separated from these samples and stored at  $-80^{\circ}\text{C}$  prior to use. Peripheral blood lymphocytes from normal controls were separated and incubated for 1 hour with these plasma samples as described in the materials and methods section. A 4 hour cytotoxicity assay was then performed using these lymphocytes against K562 target cells.

In figures 3 & 4, results of incubation of PBL's from normal healthy controls with peripheral and tumour draining venous plasma from cancer patients is shown at effector to target ratios of 50 and 25 to 1. For these experiments the control PBL's were incubated in RPMI with no added plasma.

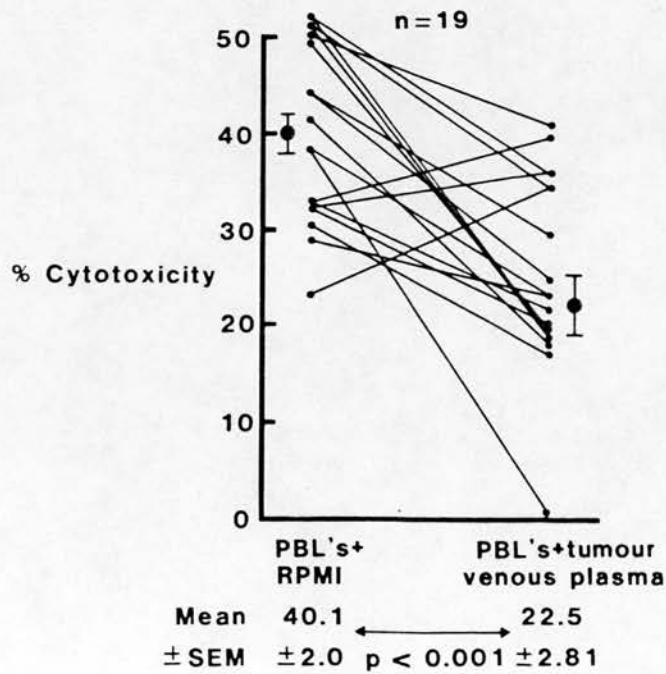
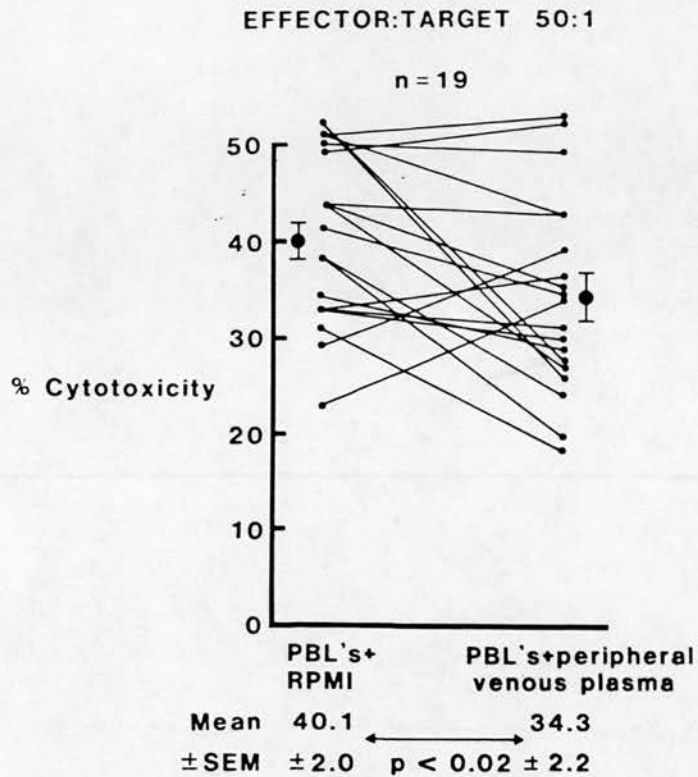
Plasma from 19 patients was available for study at the effector to target ratio of 50:1. After incubation with the peripheral venous plasma from the cancer patients, there was a reduction in the mean  $\pm$  SEM percentage cytotoxicity from  $40.1 \pm 2.0$  to  $34.3 \pm 2.2$  which was significant by the paired "t" test ( $p < 0.02$ ). Incubation of the control PBL's with the tumour venous plasma resulted in a profound depression of mean  $\pm$  SEM percentage cytotoxicity from  $40.1 \pm 2.0$  to  $22.5 \pm 2.81$  which was highly significant by the paired "t" test ( $p < 0.001$ ).

The experiment was repeated at an effector to target ratio of 25:1 and plasma from 18 patients with cancer was available for study. Incubation of the control PBL's with the peripheral venous plasma resulted in a slight reduction in mean  $\pm$  SEM percentage cytotoxicity from  $23.1 \pm 2.1$  to  $21.1 \pm 2.6$  which was not statistically significant. Incubation of the control PBL's with the tumour venous plasma, however, caused a profound depression in mean  $\pm$  SEM percentage cytotoxicity from  $23.1 \pm 2.2$  to  $10.5 \pm 1.9$  which again was highly significant by the paired "t" test ( $p < 0.001$ ).



FIGURE 3

THE INFLUENCE OF PLASMA FROM PATIENTS WITH MALIGNANT GASTROINTESTINAL TUMOURS ON NATURAL KILLING OF K562 TARGETS BY LYMPHOCYTES FROM BENIGN CONTROLS



4 hr. chromium release assay against K562 targets.

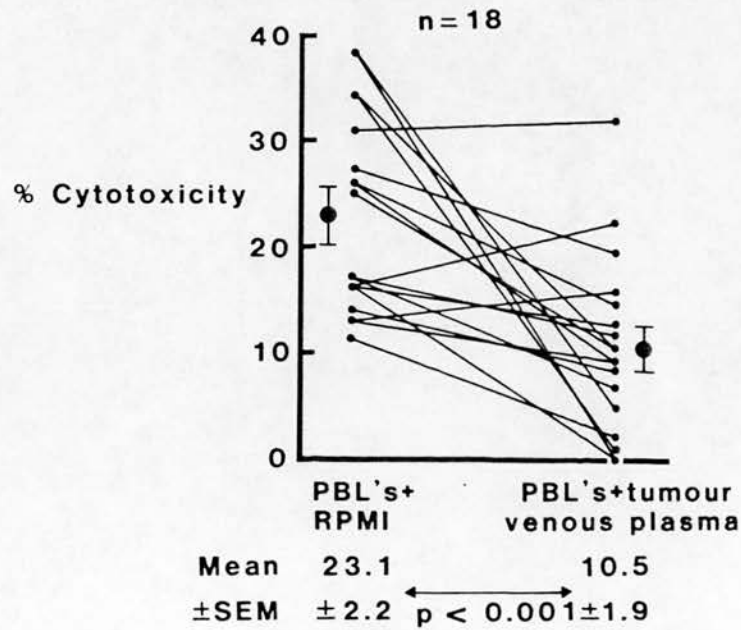
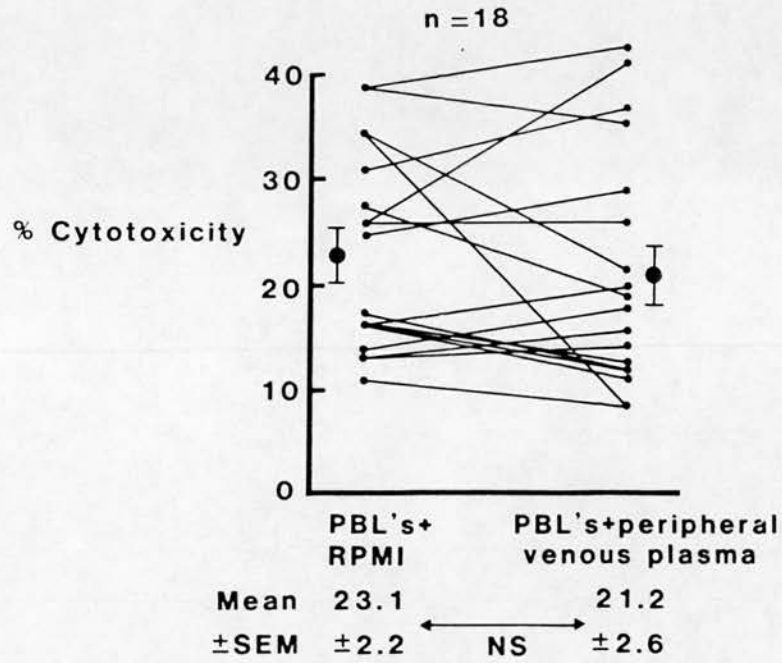
Individual patient results are the mean of 3 separate tests.

Statistical analysis is by the paired "t" test.

FIGURE 4

THE INFLUENCE OF PLASMA FROM PATIENTS WITH MALIGNANT  
GASTROINTESTINAL TUMOURS ON NATURAL KILLING OF K562  
TARGETS BY LYMPHOCYTES FROM BENIGN CONTROLS

EFFECTOR : TARGET 25 : 1



4 hr. chromium release assay against K562 targets.

Individual patient results are the mean of 3 separate tests.

Statistical analysis is by the paired "t" test.

NS = not significant

These results could be interpreted as showing evidence for a tumour factor or tumour associated factor which is in high concentration in the tumour draining venous blood. This factor could depress NK activity of normal lymphocytes but would be diluted by passage through the blood circulation which would account for the reduced level of depression seen after incubation with the peripheral venous blood of the cancer patients studied.

The results of cytotoxicity to K562 after incubation of control PBL's with plasma from the peripheral venous blood, aortic blood, tumour draining venous blood and either stomach or colon venous blood from macroscopically normal adjacent tissue are shown in Table VII with the means expressed graphically in Figure 5. Several different patterns emerge from the data expressed in Table VII and could be explained as follows:

- i. The tumour associated factor responsible for depression of natural killer activity of lymphocytes is in high concentration in the tumour draining venous blood but subsequent dilution of the factor in the circulating blood results in a lower concentration in the peripheral venous blood, aortic blood and blood draining macroscopically normal adjacent tissue, for example patients JC, JB, FP.
- ii. In some patients, for example FO, NF, DG, there are high circulating levels of this factor with a generalised reduction of cytotoxicity of control PBL's after incubation with plasma from the peripheral venous, aortic, tumour venous and blood draining adjacent normal tissue.
- iii. In some patients, e.g. WD, FW, WW the blood draining macroscopically normal tissue adjacent to the tumour appears also to depress NK activity from normal controls.

TABLE VII

THE EFFECT OF PLASMA FROM CANCER PATIENTS ON NATURAL  
CYTOTOXICITY OF PBL'S FROM NORMAL CONTROLS

Patient (colon tumours)	% Cytotoxicity				
	Control	+ PV	+ A	+ TV	+ CV
JC	51.0	53.0	46.9	18.7	44.6
RR	51.0	42.9	43.0	34.5	29.6
GW	32.7	30.0	15.6	20.2	NT
FO	38.5	24.0	24.1	23.5	21.6
DG	38.5	20.0	20.6	1.8	9.7
JB	49.3	52.9	45.3	19.8	40.0
WW	41.6	34.9	44.0	18.3	8.5
FW	44.1	35.4	25.1	1.9	1.0
NF	44.1	27.7	29.7	29.3	27.0
WD	23.2	33.9	34.9	10.3	2.9
DP	32.0	29.0	36.0	34.9	8.6
MS	50.0	49.6	41.2	44.6	48.5
AH	28.6	39.0	40.0	23.5	30.0
NW	51.6	28.4	34.2	36.2	31.2
GP	51.6	27.3	22.4	19.1	28.4
(stomach tumours)	Control	+ PV	+ A	+ TV	+ SV
WN	32.7	36.2	39.8	36.6	31.9
PW	38.5	38.1	31.2	29.7	32.3
FP	30.7	18.5	17.3	3.0	17.2
BR	32.3	31.1	21.1	21.9	0.3
Mean cytotoxicity	40.1	34.8	32.2	21.2	22.9
±S.E.M.	±2.1	±2.6	±2.3	±3.1	±3.5
P value	← $P < 0.02$ →				
by paired "t"	← $p < 0.01$ →				
with control	← $p < 0.001$ →				
	← $p < 0.001$ →				

PV = peripheral venous      A = aortic      TV = tumour venous

CV = colon venous      SV = stomach venous

4 hr. chromium release assay against K562 targets.

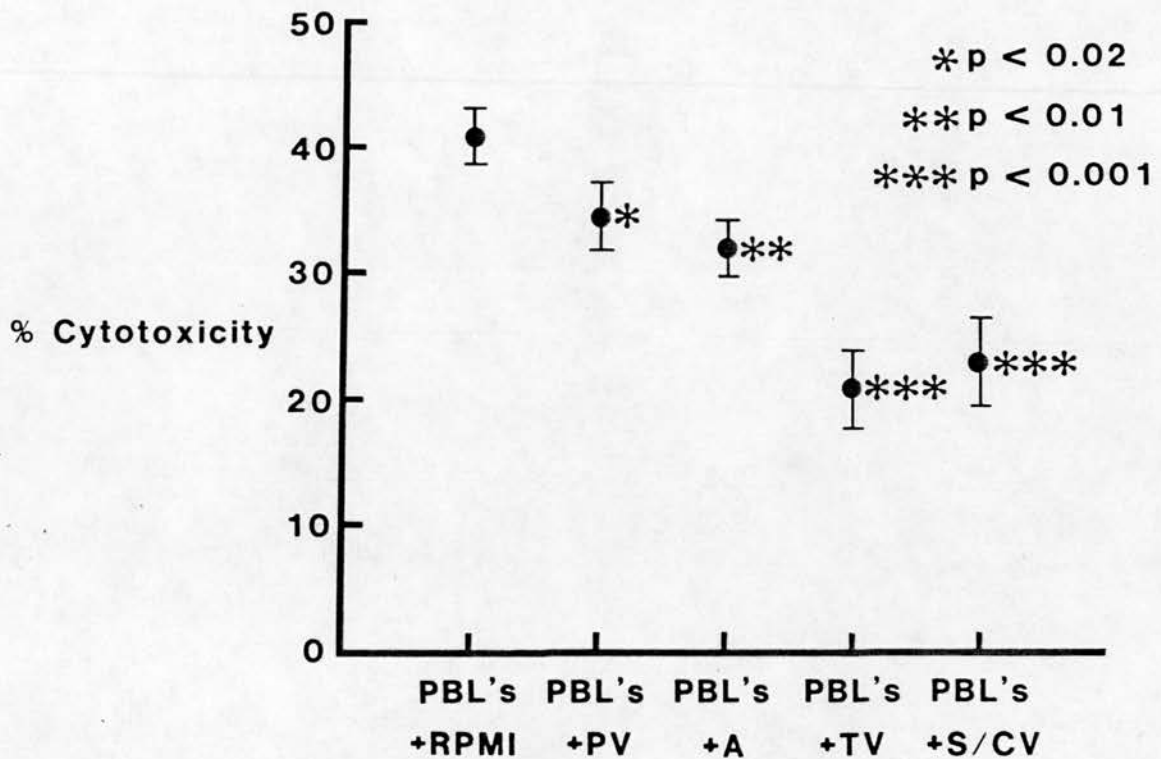
Results expressed for individual patients are the mean of 3 experiments.

Effector : target ratio 50 : 1

Statistical analysis by the paired "t" test.

**FIGURE 5**

MEAN % CYTOTOXICITY OF CONTROL PBL'S INCUBATED IN RPMI AND PLASMA  
FROM CANCER PATIENTS



PV = PERIPHERAL VENOUS PLASMA  
 A = AORTIC PLASMA  
 TV = TUMOUR VENOUS PLASMA  
 S/CV = STOMACH/COLON VENOUS PLASMA

4 hr. chromium release assay against K562 targets.

Effector to target ratio 50 : 1. Statistical analysis by paired "t" test with control PBL's in RPMI.

This could be explained by the tumour factor remaining at high concentration in the venous blood draining the adjacent tissue due perhaps to a particularly rich collateral venous drainage.

In patients, BR and DP, the plasma from the tissue adjacent to the tumour was more inhibitory to NK activity of control lymphocytes than the tumour draining venous blood, the reason for this effect is unknown.

Statistical analysis by the paired "t" test of the mean cytotoxicity of the control PBL's after incubation in the peripheral venous, aortic, tumour venous and venous blood draining adjacent tissue (stomach or colon) when compared to the control PBL's incubated in RPMI showed a significant reduction ( $p < 0.02$  -  $p < 0.001$ ) for all plasma samples. The most significant reduction was observed with the mean cytotoxicity after incubation with tumour venous plasma ( $p < 0.001$ ) and plasma from the venous blood draining adjacent tissue ( $p < 0.001$ ). These data confirm the inhibitory nature of plasma from patients with malignant gastrointestinal tumours which is most marked in the plasma from venous blood draining the tumour and tissue adjacent to the tumour.

#### 4.8 The effect of plasma from patients with benign abdominal disease on natural killing of K562 targets by lymphocytes from normal controls.

This experiment was undertaken to establish whether the stomach or colon per se in patients with non malignant disease could produce factors which might be inhibitory to natural cytotoxicity of lymphocytes.

In 6 patients with benign upper abdominal conditions undergoing routine surgical procedures, plasma was separated from the aortic blood, venous blood draining the stomach and venous blood draining the colon. Fifty



microlitres of PBL's from normal subjects of a concentration of  $1 \times 10^6$  cells/ml and  $0.5 \times 10^6$  cells/ml were incubated with 50 $\mu$ L of plasma for 1 hour at 37°C prior to use in a 4 hour cytotoxicity assay against K562 target cells at an effector to target ratio of 50:1 and 25:1. The patient details and results of the cytotoxicity assays are shown in Table VIII.

At the 50:1 effector to target ratio studied, the plasma from the aortic, stomach venous and colon venous blood of 3 patients (WW, PB, MB) failed to inhibit natural killing by control PBL's. Plasma from the stomach draining venous blood of 2 patients with histologically benign gastric ulcer (FA, MT) did however depress natural cytotoxicity of the control PBL's and plasma from the stomach and colon draining venous blood of one patient (HB) caused depression of natural cytotoxicity. The difference in the mean  $\pm$  SEM percentage cytotoxicity of the control PBL's at  $35.3 \pm 7.1\%$  and the PBL's incubated in aortic plasma at  $33.8 \pm 6.3$  was not significant. The mean  $\pm$  SEM cytotoxicities of the PBL's incubated in stomach venous plasma ( $20.5 \pm 7.9\%$ ) and colon venous plasma ( $27.8 \pm 7.5\%$ ) was significantly reduced ( $p < 0.05$  and  $p < 0.02$ ) when compared to the control PBL's at this effector to target ratio.

At the 25:1 effector to target ratio, the plasma from the stomach draining venous blood of one patient with a gastric ulcer (MT) caused depression of natural cytotoxicity of the control PBL's to K562 targets as did plasma from the colon venous blood of patient HB.

The difference in the mean  $\pm$  SEM cytotoxicities of the PBL's incubated in aortic plasma ( $18.3 \pm 3.5\%$ ), stomach venous plasma ( $14.3 \pm 4.6\%$ ) and colon venous plasma ( $16.3 \pm 4.3\%$ ) did not reach statistical significance when compared to the mean  $\pm$  SEM percentage cytotoxicity of the control PBL's ( $18.7 \pm 3.0\%$ ).

TABLE VIII

**THE EFFECT OF PLASMA FROM PATIENTS WITH BENIGN DISEASE  
ON PERCENTAGE CYTOTOXICITY OF PBL'S FROM NORMAL CONTROLS  
TO K562 TARGET CELLS**

Effector : Target ratio 50 : 1

Patient	Diagnosis	% cytotoxicity	% cytotoxicity of PBL's + plasma		
		Control PBL's	A	SV	CV
WW	D U	24.3	22.2	24.1	20.4
PB	Gallstones	24.3	22.2	15.5	21.1
MB	Gallstones	55.0	57.0	57.0	55.0
FA	G U	55.0	49.2	18.3	38.0
MT	G U	40.0	31.4	5.7	32.0
HB	Gallstones	13.2	20.9	2.5	0.7
	Mean	35.3	33.8	20.5	27.8
	±S.E.M.	±7.1	±6.3	±7.9	±7.5
	p value	<div style="text-align: center;"> <math>\xleftrightarrow{\text{NS}}</math>  <math>\xleftrightarrow{p &lt; 0.05}</math>  <math>\xleftrightarrow{p &lt; 0.02}</math> </div>			

Effector : target ratio 25 : 1

Patient	Diagnosis	% cytotoxicity	% cytotoxicity of PBL's + plasma		
		Control PBL's	A	SV	CV
WW	D U	14.4	11.8	13.4	14.2
PB	Gallstones	14.4	13.2	9.9	12.7
MB	Gallstones	27.0	32.0	33.8	33.3
FA	G U	27.0	25.9	19.7	17.3
MT	G U	20.6	19.4	9.3	19.8
HB	Gallstones	9.0	10.1	—	0.6
	Mean	18.7	18.3	14.3	16.3
	±S.E.M.	±3.0	±3.5	±4.6	±4.3
	p value	<div style="text-align: center;"> <math>\xleftrightarrow{\text{NS}}</math>  <math>\xleftrightarrow{\text{NS}}</math>  <math>\xleftrightarrow{\text{NS}}</math> </div>			

4 hr. chromium release assay against K562 targets.

Individual patient results are the mean of 3 experiments.

A = aortic plasma    SV = stomach venous plasma    CV = colon venous plasma

Statistical analysis by the paired "t" test

NS = not significant

The depression of natural cytotoxicity seen when control PBL's were incubated in the stomach venous plasma of 2 patients with benign gastric ulcers is of interest since although these ulcers were histologically benign, they could in some way be responsible for the production of a factor by inflammatory cells reacting to the ulcer which could depress NK activity. In patient HB, the stomach and colon venous plasma was inhibitory to NK activity of the PBL's at the effector to target ratios of 50:1, no explanation can be put forward for this effect.

Taking the results overall, apart from the patients with gastric ulcers, it would appear that normal stomach and colon in patients with benign gastrointestinal disease, do not consistently produce factors which result in depression of natural killer cell activity by PBL's from normal controls. The number of patients studied however, was small and more patients should be studied in future to determine this with certainty.

#### 4.9 The effect of heat treatment of plasma from patients with benign disease on natural cytotoxicity of PBL's from normal controls.

Plasma from 5 patients with benign abdominal disease (cholelithiasis) was heated at 56°C for 40 minutes and incubated with PBL's from normal controls prior to cytotoxicity assay against K562 targets. This was compared to the cytotoxicity of PBL's incubated with non heat treated plasma from the same patients (Table IX). This was a statistically significant ( $p < 0.005$ ) fall in mean  $\pm$  SEM percentage cytotoxicity from  $17.3 \pm 1.25\%$  after incubation with the non heated plasma to  $11.6 \pm 2.0\%$  for the PBL's incubated with heat treated plasma indicating that heat treatment of the plasma had altered the plasma to cause depression of NK activity of normal PBL's.

TABLE IX

THE EFFECT OF HEAT TREATMENT OF PLASMA FROM BENIGN  
CONTROLS ON PERCENTAGE CYTOTOXICITY OF PBL'S FROM  
BENIGN CONTROLS

Effector : target ratio 25 : 1

% cytotoxicity to K562

	Control PBL's + RPMI	Control PBL's + unheated plasma	Control PBL's + heat treated plasma
	18.8	18.0	9.1
	18.8	19.2	17.1
	18.8	16.9	13.4
	18.8	12.6	5.1
	18.8	19.9	13.5
Mean	18.8	NS → 17.3	p < 0.005 → 11.6
±S.E.M.	±0	±1.25	p < 0.005 → ±2.0

4 hr. chromium release assay against K562 targets.

Individual results are the mean of 3 experiments.

Statistical analysis by the paired "t" test.

NS = not significant.

4.10 The effect of heat treatment of plasma from patients with malignant disease on natural cytotoxicity of PBL's from normal controls.

Previous experiments had shown that the cytotoxicity of PBL's from normal controls was reduced after incubation with plasma from the peripheral venous blood of patients with cancer and the cytotoxicity of these lymphocytes was profoundly reduced after incubation with the plasma from the venous blood draining the tumour.

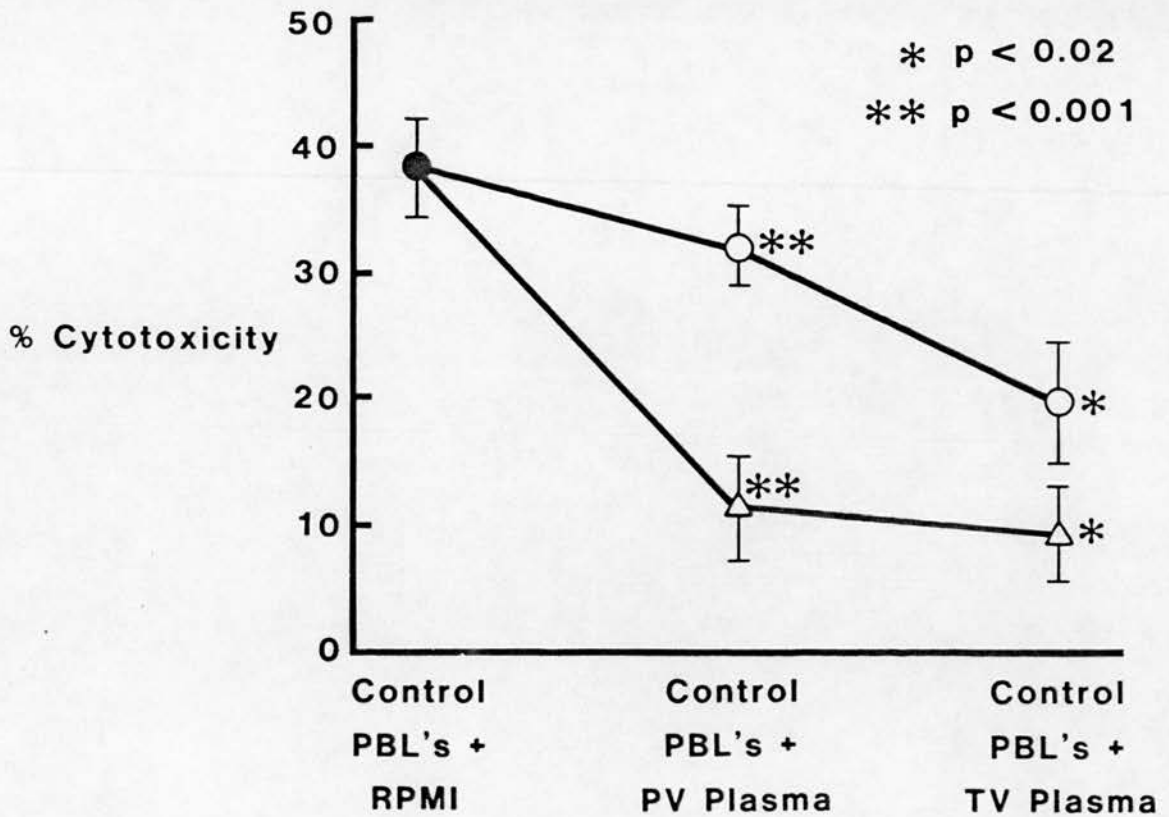
This could suggest the presence of a tumour factor or tumour associated factor in high concentration in the tumour venous blood. In this experiment, the effect of heat inactivation of plasma from the cancer patients was assessed. Plasma was heated at  $56^{\circ}\text{C}$  for 40 minutes to determine whether the factor responsible for depression of NK activity of control PBL's was heat stable.

Heat treatment of plasma from the peripheral venous and tumour venous blood of 9 patients with gastrointestinal cancer resulted in profound depression of natural killing of control lymphocytes ( $11.4 \pm 3.9\%$  and  $9.8 \pm 3.5\%$ ) compared to the non heat inactivated plasma from the same sites ( $31.8 \pm 3.2\%$   $p < 0.001$  and  $19.9 \pm 4.7\%$   $p < 0.02$ ) respectively. (Figure 6).

These results suggest that the depressant factor found in tumour draining venous blood may be heat stable because if the factor were inactivated by heat treatment of the plasma, either abrogation or a substantial reduction in the depression of cytotoxicity of control lymphocytes incubated in the heat treated tumour venous plasma would be expected.

**FIGURE 6**

THE EFFECT OF HEAT TREATMENT OF PLASMA FROM CANCER PATIENTS ON CYTOTOXICITY  
OF CONTROL PBL'S TO K562 TARGETS  
EFFECTOR : TARGET 50 : 1



4 hr. chromium release assay against K562 targets using PBL's from a healthy control.

Effector : target ratio 50 : 1

○ = non heat treated plasma

△ = heat treated plasma

Mean cytotoxicity for PBL's incubated in heat treated and non heat treated plasma obtained from 9 patients.

Statistical analysis is by paired "t" test.



The reason for the depressed level of cytotoxicity of the control PBL's seen after incubation with the heat treated plasma remains unknown but may be related to precipitates of plasma proteins being formed which may block NK activity of the control lymphocytes by inhibiting binding to tumour target cells.

#### 4.11 Assay for immune complexes in peripheral venous and tumour draining venous blood.

Circulating immune complexes have been identified in cancer patients by many workers and the levels of these complexes may be related to the prognosis of these patients. These complexes are thought to result from the shedding of tumour associated antigen which complexes with IgG or IgM and therefore these complexes would be expected to be in high concentration in the venous blood draining the tumour.

One suggested mechanism of depressed natural killer lymphocyte cytotoxicity to tumour cells by tumours is the combination of the immunoglobulin associated with the immune complex with the Fc immunoglobulin receptor on the NK lymphocyte which could result in reduced binding of NK cells to tumour cells. Timonen and Saksela (1977) have demonstrated that NK cell killing of target cells is reduced in the presence of immune complexes of IgG and IgM.

The Raji cell assay is one of the most sensitive assays for circulating immune complexes described for patients with colorectal tumours (Hobbiss et al 1983). This assay was performed using the tumour venous plasma from 11 cancer patients to detect possible presence of high levels of circulating immune complexes.

In Table X the results of Raji cell assays for immune complexes in the plasma of peripheral and tumour venous blood of 11 patients are shown together with their effect on NK cell cytotoxicity towards K562 targets. The results are expressed as  $\mu\text{g/ml}$  of Cohn fraction II, values above  $20 \mu\text{g/ml}$  are regarded as positive for immune complexes.

None of the 11 patients studied showed evidence of levels of immune complexes in the tumour venous or peripheral venous blood above the normal level of laboratory controls. It remains possible that immune complexes were present but not detected by the Raji cell assay. This, however, is unlikely in view of the sensitivity of this assay in patients with cancer found by other workers. These results would therefore suggest that the depression in natural cytotoxicity of lymphocytes to K562 target cells is not mediated by the presence of immune complexes.

#### 4.12 Studies of white blood cell counts by morphology across the tumour circulation.

These studies were undertaken to determine the total white blood cell counts, polymorphonuclear leucocyte counts and total lymphocyte counts in the arterial blood entering the tumour and the venous blood draining the tumour.

##### a. Total white blood cell counts.

Aortic blood, representing arterial blood entering the tumour, and the venous blood draining the tumour was prepared and stained as described in the materials and methods section for microscopy.

The results for the total white blood cell counts were expressed as cells  $\times 10^9/\text{L}$  and are shown in Figure 7 (a). Although 2 patients showed marked reduction of total white blood cell counts across the

TABLE X

**RAJI CELL ASSAY FOR CIRCULATING IMMUNE COMPLEXES IN  
TUMOUR VENOUS AND PERIPHERAL VENOUS BLOOD OF  
PATIENTS WITH CANCER**

Patient	Site of tumour	TV plasma effect on cytotoxicity on control PBL's	Raji cell assay TV plasma	Raji cell assay PV plasma
FP	Stomach	depressed	2.5	2.7
PW	Stomach	no change	2.2	1.5
JB	Colon	depressed	1.9	2.4
RR	Colon	depressed	1.6	1.6
FW	Colon	depressed	1.7	1.6
NW	Colon	depressed	4.1	4.9
AP	Colon	depressed	3.1	4.8
WN	Stomach	no change	3.2	3.4
DG	Colon	depressed	3.4	3.8
WD	Colon	depressed	4.4	4.2
BR	Stomach	depressed	5.5	4.2

Results are expressed in  $\mu\text{g/ml}$

Values above 20  $\mu\text{g/ml}$  are considered positive for immune complexes

tumour circulation (i.e. aortic to tumour venous) the majority of patients showed no significant change and the difference between the mean  $\pm$  SEM count for the aortic blood at  $6 \pm 0.72 \text{ cells} \times 10^9/\text{L}$  and the mean  $\pm$  SEM for the tumour venous blood at  $5 \pm 0.54 \times 10^9/\text{L}$  was not statistically significant by the paired "t" test.

b. Total lymphocyte counts.

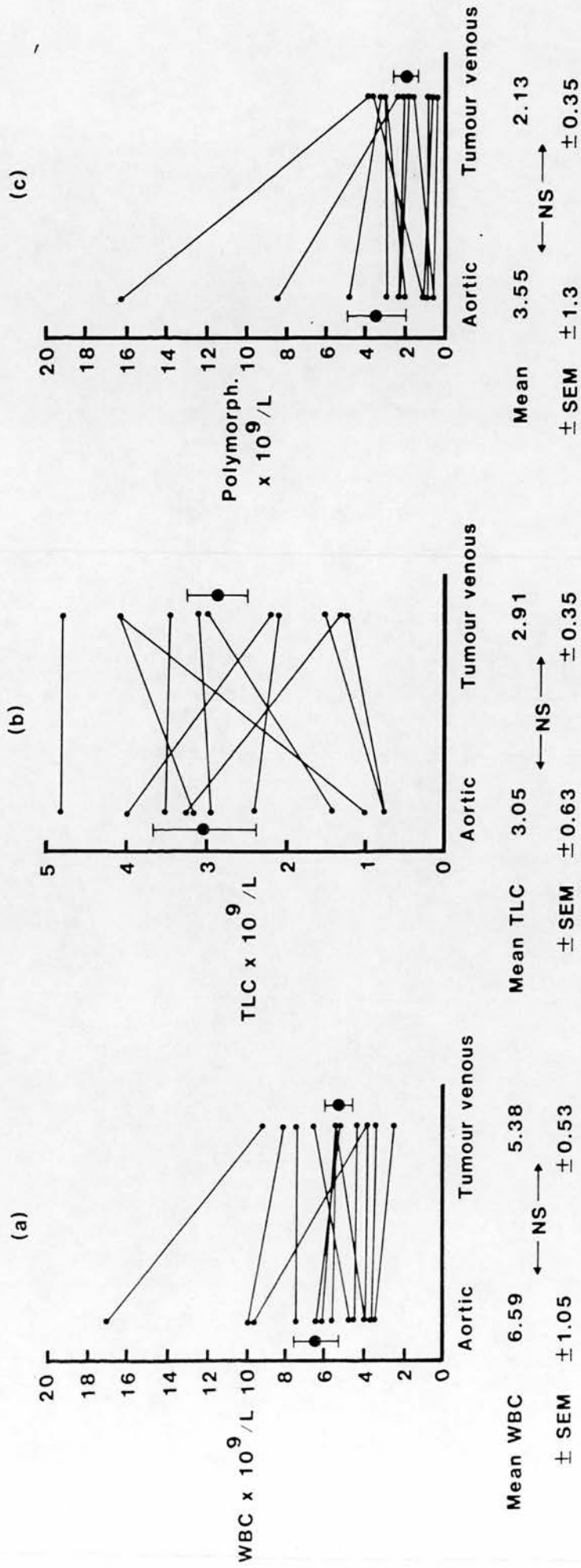
Total lymphocyte counts by microscopy were performed on aortic and tumour draining venous blood and expressed as cell  $\times 10^9/\text{L}$ . The results are shown in Figure 7 (b). Three patients showed considerable variation of total lymphocyte counts across the tumour circulation, one with elevated numbers in the tumour venous blood and two with reduced numbers in the tumour venous blood when compared to the aortic blood. The remaining patients did not show any marked difference in total lymphocyte counts across the tumour circulation and this is reflected in the lack of a statistically significant difference between the mean  $\pm$  SEM total lymphocyte count of the aortic blood at  $3.05 \pm 0.63 \times 10^9 \text{ cells/ml}$  and the mean  $\pm$  SEM of the tumour venous blood at  $2.91 \pm 0.35 \times 10^9 \text{ cells/ml}$ .

c. Polymorphonuclear leucocyte counts.

Polymorphonuclear leucocyte counts were performed on aortic and tumour draining venous blood with counts expressed as cells  $\times 10^9/\text{mL}$ . The results are shown in Figure 7 (c). Two patients showed considerable reduction in polymorphonuclear leucocyte cell counts across the tumour circulation and these 2 patients correspond to the 2 patients in Figure 7 (a) who demonstrated a considerable reduction in total white blood cell count across the tumour indicating that this fall was due to a reduction in polymorphonuclear leucocyte numbers. This may represent sequestration of these cells in the tumour at a site of

FIGURE 7

TOTAL WHITE BLOOD CELL COUNTS AND TOTAL LYMPHOCYTE COUNTS AND POLYMORPHONUCLEAR LEUCOCYTES ACROSS THE TUMOUR BY MORPHOLOGY



Statistical analysis by the paired "t" test.

NS = not significant.

Cell counts by microscopy after Giemsa staining



bacterial inflammation. In 2 patients there was elevation of polymorphonuclear leucocyte numbers across the tumour however the majority of patients failed to show any appreciable change across the tumour. The mean  $\pm$  SEM aortic polymorphonuclear leucocyte cell count at  $3.55 \pm 1.3 \times 10^9$  cells/ml was not significantly different from the tumour venous count at  $2.13 \pm 0.35 \times 10^9$  cells/L but perhaps suggests a net sequestration of polymorphonuclear leucocytes within the tumour.

#### 4.13 Studies using the monoclonal antibody Leu 7 on lymphocytes traversing the tumour circulation.

A possible mechanism of depressed natural killer lymphocyte activity of blood from patients with cancer is the sequestration of natural killer lymphocytes within the tumour. Experiments were therefore performed to identify NK lymphocyte populations across the tumour circulation using the monoclonal antibody Leu 7. This antibody is a relatively specific marker for the natural killer lymphocyte although there is some cross reactivity with suppressor lymphocytes.

There were sufficient lymphocytes available from the aortic blood and tumour draining venous blood of 9 patients with gastrointestinal tumours for labelling with Leu 7 monoclonal antibody (Abo and Balch 1981).

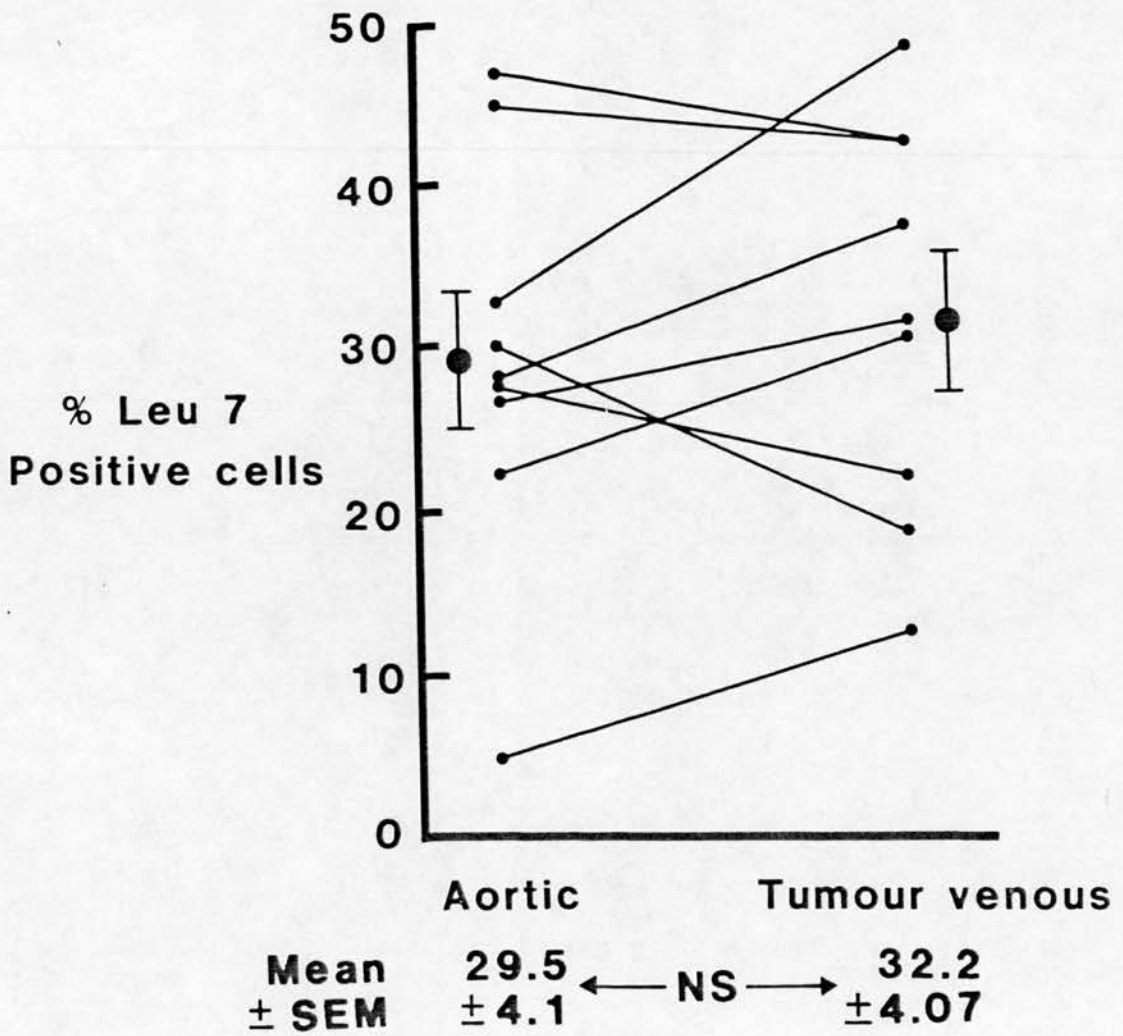
Lymphocytes were separated and labelled with the Leu 7 antibody using an individual fluorescent technique as described in the materials and methods section. Ultraviolet microscopy was performed to determine the percentage of Leu 7 positive cells in each lymphocyte population.

The results (Figure 8) show that there is some individual tumour variation with a reduction in the percentage of Leu 7 positive cells after passage



**FIGURE 8**

**% LEU 7 POSITIVE CELLS (NK) ACROSS THE TUMOUR BY INDIRECT  
MEMBRANE IMMUNO FLUORESCENCE**



Statistical analysis by the paired "t" test.

NS = not significant

TABLE XI

PERCENTAGE OF LEU 7 POSITIVE CELLS ACROSS THE TUMOUR  
CIRCULATION COMPARED TO THE EFFECT OF PASSAGE THROUGH  
THE TUMOUR CIRCULATION ON NK CYTOTOXICITY OF WHOLE BLOOD

Patient	Tumour	Aortic	Tumour venous	Cytotoxicity to K562 after passage through the tumour circulation
FW	Colon	33%	49%	depressed
WW	Colon	45%	43%	elevated
MS	Colon	5%	13%	elevated
NF	Colon	47%	43%	no change
HW	Stomach	27%	32%	elevated
AH	Colon	27.5%	22.2%	elevated
AP	Colon	28.3%	38.1%	depressed
NW	Colon	22.8%	31.1%	no change

through the tumour circulation in 4 patients and an elevated percentage of Leu 7 positive cells in 5 patients. The difference between the mean  $\pm$  SEM percentage of Leu 7 positive cells in the aortic blood at  $29.5 \pm 4.1\%$  and that of the tumour venous blood at  $32.3 \pm 4.07\%$  was not statistically significant by the paired "t" test.

In Table XI, the percentage of natural killer lymphocytes as determined by positive staining with Leu 7 monoclonal antibody in the arterial blood entering the tumour circulation and the venous blood draining the tumour with the net effect on the cytotoxicity of whole blood to K562 target cells after passage through the tumour circulation is shown in 8 patients. There appeared to be no correlation between reduced percentage of Leu 7 positive cells and alteration in NK lymphocyte cytotoxicity of whole blood after passage through the tumour circulation.

This observation would suggest that alteration of natural cytotoxicity of whole blood after passage through the tumour is not related to changes in NK lymphocyte numbers. In those tumours where depression of natural cytotoxicity is observed in the tumour draining venous blood, this effect is likely to be due to an effect on NK lymphocytes, resulting in reduced capacity for natural killing of tumour cells rather than depletion of cell numbers.

#### 4.14 Discussion.

From the results of the experiments described in this chapter, it would appear that malignant tumours of the gastrointestinal tract have an effect on the natural cytotoxicity to tumour cells mediated by the natural killer lymphocytes of the host. Patients with malignant disease localised to the organ of origin of the primary tumour did have reduced natural

cytotoxicity when compared to benign controls however this difference was not statistically significant with the number of patients studied. Those patients with disseminated malignant disease with evidence of distant tumour metastases did have profoundly reduced natural cytotoxicity when compared to the benign controls. This would suggest that depression of NK lymphocyte activity in patients with malignant gastrointestinal tumours is directly related to tumour burden.

The effect of passage of blood through the tumour circulation on natural cytotoxicity showed no statistically significant depression of cytotoxicity in the tumour draining venous blood in the majority of patients. It may be that the speed of transit of a natural killer lymphocyte in whole blood through the tumour circulation from the arterial to venous side is too rapid (in the order of several seconds only) for depressant factors produced by the tumour to have sufficient time to act on the NK lymphocytes. Further evidence, however, that malignant tumours of the gastrointestinal tract directly influence natural killer cell activity was found after incubation for 1 hour of lymphocytes from normal control subjects with plasma from cancer patients. Plasma from the peripheral venous blood of the cancer patients had a moderate depressant effect on cytotoxicity to K562 target cells and plasma from the tumour draining venous blood caused profound depression of cytotoxicity strongly suggesting the production of a depressant factor which could inhibit innate natural cytotoxicity of the host to metastatic tumour formation. This factor appeared to be heat stable and not a result of immune complex formation in the tumour. This depressant factor was not produced by the stomach or colon of patients with benign disease giving further support for the production of a tumour or tumour associated factor, present in high concentration in the tumour draining venous blood and possibly also the

venous blood draining adjacent macroscopically normal stomach or colon, which depress natural cytotoxicity of lymphocytes to tumour target cells.

Investigation of the white blood cell populations across the tumour have suggested that some tumours were sequestering white blood cells, mainly polymorphonuclear leucocytes however there was no convincing evidence of sequestration of Leu 7 NK lymphocytes to account for the depressed NK lymphocyte activity found in some patients with cancer.

CHAPTER 5.



## CHAPTER 5.

Experimental results relating to the behaviour of lymphocytes during anaesthesia and surgery.

### 5.1 Introduction.

5.2 The cytotoxicity in vitro against K562 target cells of natural killer lymphocytes from patients with benign and malignant disease during surgery under infusion anaesthesia.

5.3 The cytotoxicity in vitro against K562 target cells of natural killer lymphocytes from patients with benign and malignant disease during surgery under inhalational anaesthesia.

5.4 Natural cytotoxicity against K562 targets in patients with disseminated malignant disease during surgery. Comparison with patients with local malignant disease.

5.5 Cytotoxicity against K562 targets of peripheral blood taken before anaesthesia, during anaesthesia alone and during surgery in patients with benign and malignant disease.

5.6 Total white blood cell counts, polymorphonuclear leucocyte counts and total lymphocyte counts before and during surgery in patients with benign disease.

5.7 Total white blood cell counts, polymorphonuclear leucocyte counts and total lymphocyte counts before and during surgery in patients with cancer.

- 5.8 Analysis of lymphocyte subpopulations in cancer patients before and during surgery using monoclonal antibody (Leu 7).
- 5.9 The percentage of Leu 7 positive cells in the peripheral venous blood pre anaesthesia, pre surgery and during surgery in cancer patients.
- 5.10 Cytotoxicity of whole blood towards K562 target cells, during surgery and 48 hours following surgery in patients with benign abdominal disease.
- 5.11 Cytotoxicity of whole blood towards K562 target cells, during surgery and 48 hours following surgery in patients with malignant abdominal disease.
- 5.12 Plasma interferon levels before and during surgery in patients with benign disease and cancer demonstrating elevated natural cytotoxicity during surgery.
- 5.13 The effect of exogenous interferon in vitro on natural cytotoxicity towards K562 targets of lymphocytes taken during surgery in patients with benign disease and cancer.
- 5.14. Discussion.

## 5.1 Introduction.

The main treatment modality of patients with potentially curable gastrointestinal cancer is surgical resection of the tumour under general anaesthesia . Natural killer (NK) lymphocytes have been shown, in experimental animals, to actively destroy tumour cells injected intravenously (Riccardi et al 1979) and this evidence infers a role for the NK lymphocyte in defence against tumour metastases. The experiments described in this chapter were designed to study the cytotoxicity of natural killer cells to tumour target cells in vitro from patients with benign disease and cancer to determine whether general anaesthesia or surgical operation adversely affects the behaviour of this limb of the immune defence system, which may play a critical role in preventing tumour spread via the blood and hence prevent the development of metastases.

## 5.2 The cytotoxicity in vitro against K562 target cells of natural killer lymphocytes from patients with benign and malignant disease during surgery under infusion anaesthesia.

In 11 patients with benign upper abdominal disease and 6 patients with malignant disease confined to either the stomach or colon, definitive surgical operations were performed under intravenous infusion anaesthesia using Etomidate and Fentanyl (Jones et al 1983). This anaesthetic technique is currently under evaluation and therefore strict criteria for patient selection were exercised by the anaesthetists so that no patient over 65 years of age received this type of anaesthesia. Five millilitres of venous blood was taken by peripheral venepuncture into heparinized tubes before induction of anaesthesia and again one hour

after induction of anaesthesia and during the definitive surgical procedure. These blood samples were used in a whole blood cytotoxicity assay against K562 target cells and the results expressed as percentage cytotoxicity (Figure 1).

Of the 11 patients with benign disease, 8 showed elevation of percentage cytotoxicity of whole blood during the surgical procedure compared to blood taken before anaesthesia, one patient showed no change and in 2 patients, percentage cytotoxicity was depressed during the surgical procedure. The mean percentage cytotoxicity  $\pm$  SEM rose from  $18.9 \pm 4.1$  before anaesthesia to  $23.7 \pm 3.1$  during surgery, on statistical analysis of the grouped data the difference was not significant (by paired "t" test).

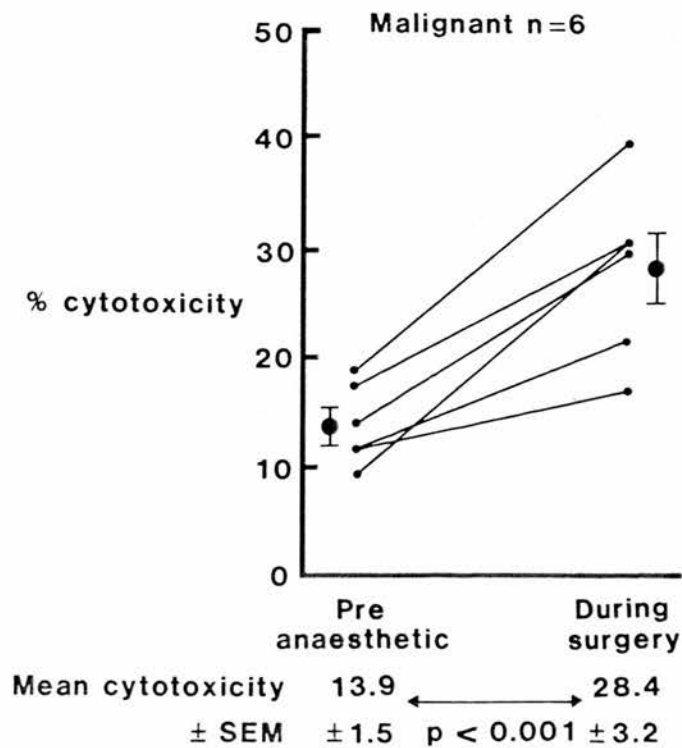
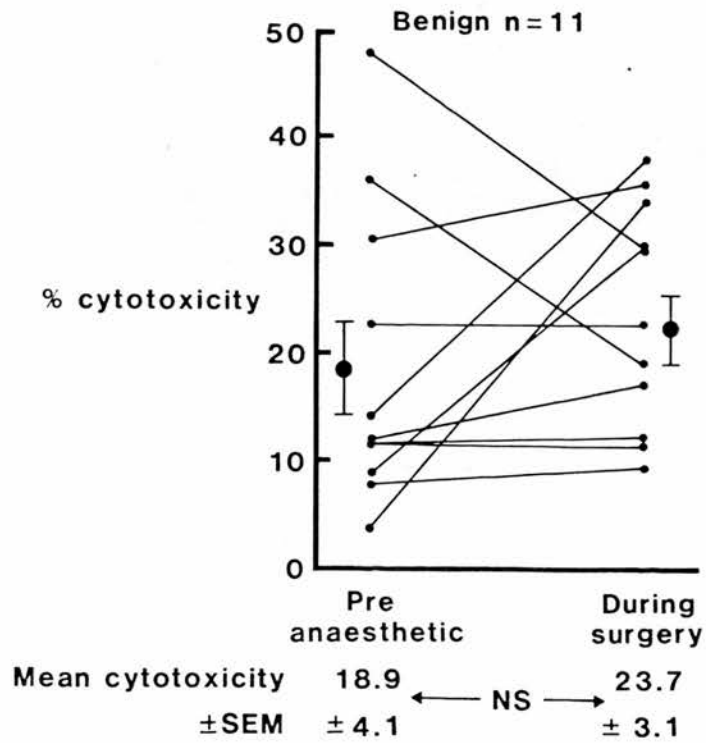
The 6 patients with malignant disease who had their definitive surgical procedure under infusion anaesthesia all showed a marked rise in percentage cytotoxicity during surgery. The mean percentage cytotoxicity  $\pm$  SEM rose from  $13.9 \pm 1.5$  before anaesthesia to  $28.4 \pm 3.2$  during anaesthesia and was shown to be highly significant by the paired "t" test ( $p < 0.001$ ).

### 5.3 The cytotoxicity in vitro against K562 target cells of natural killer lymphocytes from patients with benign and malignant disease during surgery under inhalation anaesthesia.

Nine patients with benign upper abdominal disease and 12 patients with malignant disease confined to the stomach or colon had definitive surgical operations performed under conventional inhalational anaesthesia, using nitrous oxide, halothane and oxygen. Five millilitres of blood was taken from a peripheral vein before the induction of anaesthesia and during the surgical procedure. These blood samples were used in a whole

FIGURE 1

PERCENTAGE CYTOTOXICITY TO K562 TARGETS OF PATIENTS  
WITH BENIGN AND MALIGNANT DISEASE DURING SURGERY  
UNDER INFUSION ANAESTHESIA



4 hr. chromium release assay of whole blood against K562 target.

$1/2$  dilution with RPMI.

Statistical analysis by the paired "t" test.

blood cytotoxicity assay against K562 target cells and the results expressed as percentage cytotoxicity (Figure 2).

Six of the 9 patients with benign abdominal disease showed elevated levels of percentage cytotoxicity during surgery. One of the remaining 3 patients showed depression of cytotoxicity during surgery and 2 patients showed no change in cytotoxicity during surgery. The rise in mean  $\pm$  SEM cytotoxicity for the benign patients from  $11.9 \pm 2$  before induction of anaesthesia to  $21.8 \pm 4.5$  during surgery was not found to be significant by the paired "t" test.

Twelve patients with malignant tumours confined to the stomach or colon, had definitive surgical procedures under conventional inhalational anaesthesia. Eight of the 12 patients displayed elevated levels of percentage cytotoxicity to K562 target cells during surgery and this was reflected in a rise in the mean  $\pm$  SEM cytotoxicity for the group from  $9.8 \pm 2.0$  to  $12.7 \pm 1.85$ . This was statistically significant when analysed by the paired "t" test ( $p < 0.01$ ).

#### 5.4 Natural cytotoxicity against K562 targets, in patients with disseminated disease during surgery. Comparison with patients with local malignant disease.

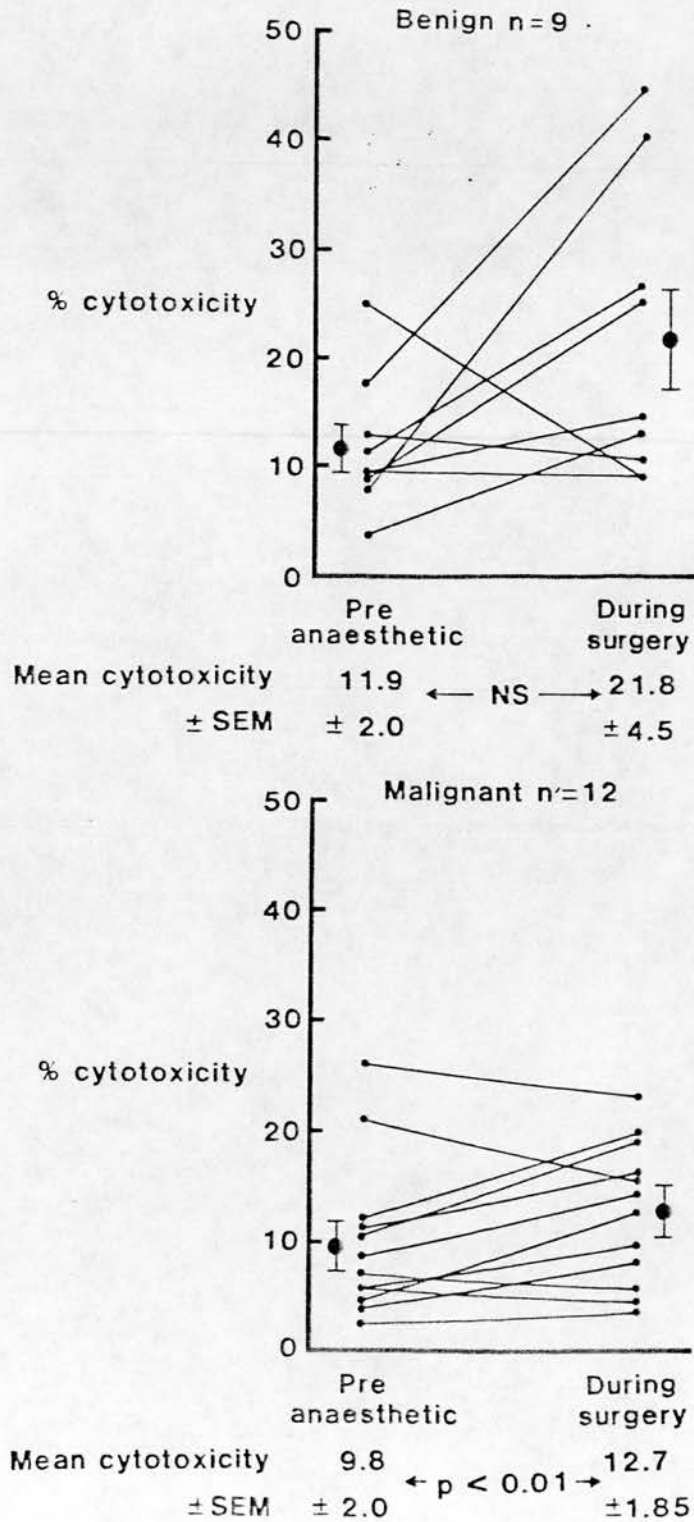
The 8 patients with disseminated malignant disease were considered separately since it was known that this group of patients had significant reduction of natural killer lymphocyte activity compared to patients with malignant disease confined either to the stomach or colon. (See chapter 4).

Five of the 8 patients with disseminated malignancy had elevation of cytotoxicity during the palliative surgical procedure with 2 patients showing depression and the remaining patient showing no change of



FIGURE 2

PERCENTAGE CYTOTOXICITY TO K562 TARGETS OF PATIENTS  
WITH BENIGN AND MALIGNANT DISEASE DURING SURGERY  
UNDER INHALATIONAL ANAESTHESIA



4 hr. chromium release assay for whole blood against K562 targets.

$1/2$  dilution with RPMI.

Statistical analysis by the paired "t" test.

cytotoxicity during surgery. Seven of these patients received conventional inhalational anaesthesia during surgery and one patient had surgery performed under infusion anaesthesia with Etomidate and Fentanyl. The mean  $\pm$  SEM rise in percentage cytotoxicity from the pre anaesthetic level of  $5.8 \pm 1.5$  to  $8.3 \pm 2.3$  was not significant by the paired "t" test.

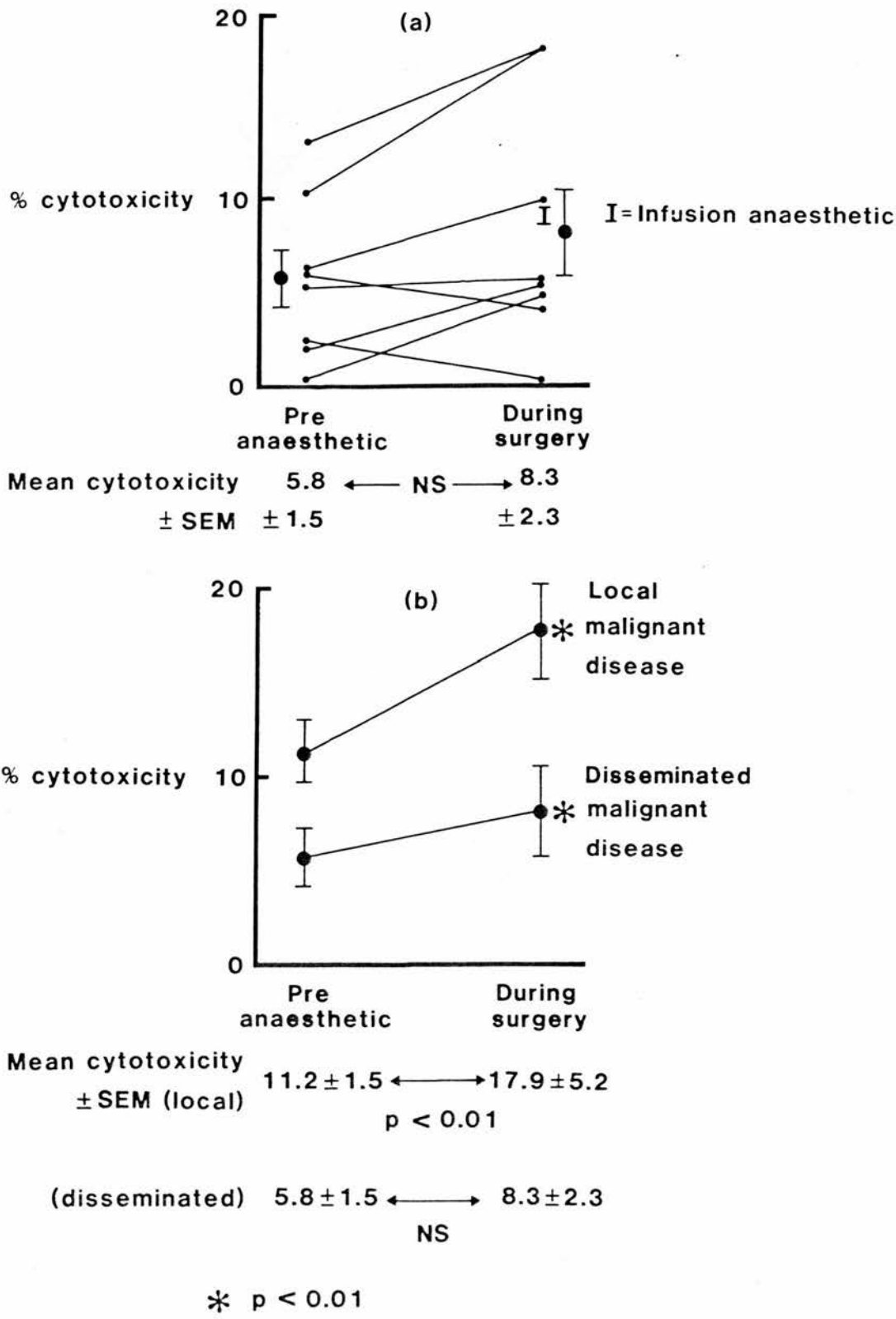
In figure 3b, the mean cytotoxicity of all of the patients with local malignant disease receiving both infusion and inhalational anaesthesia before and during the surgical procedure is shown, compared to the mean cytotoxicity of patients with disseminated malignant disease before and during surgery. The mean cytotoxicity of the patients with disseminated malignancy taken before induction of anaesthesia, and also during surgery is significantly lower ( $p < 0.01$ ) than the mean cytotoxicity of the patients with localised tumours with blood samples taken before and during surgery. The magnitude of the rise in mean cytotoxicity of the patients with localised tumours is also greater during surgery than the patients with disseminated disease, which suggests that the NK lymphocytes from patients with a large tumour burden with widespread metastatic disease may be less responsive to factors operating during the surgical procedure under general anaesthesia which tend to enhance natural killer lymphocyte activity.

#### 5.5 Cytotoxicity, against K562 targets, of peripheral blood taken before anaesthesia, during anaesthesia alone and during surgery in patients with benign and malignant disease.

Previous experiments had demonstrated elevated levels of cytotoxicity to K562 target cells during the surgical procedure under general anaesthesia

FIGURE 3

PERCENTAGE CYTOTOXICITY AGAINST K562 TARGETS IN PATIENTS WITH DISSEMINATED MALIGNANT DISEASE DURING SURGERY. COMPARISON WITH PATIENTS WITH LOCAL MALIGNANT DISEASE



Comparing means pre anaesthetic and means during surgery by student's "t" test.  
The rise in mean cytotoxicity during surgery for patients with local malignant disease is significant (p < 0.01) by the paired "t" test.

(infusion and inhalation), therefore further experiments were undertaken to "dissect" the nature of this response into events before induction of anaesthesia, during anaesthesia alone (prior to surgery) and during the surgical procedure under anaesthetic. Five ml of peripheral venous blood was taken from 9 patients with benign disease and 17 patients with localised gastrointestinal cancer before induction of anaesthesia, 10 minutes after induction of anaesthesia and before the skin incision, and again 1 hour after induction of anaesthesia during the surgical operation. These samples were used in a 4 hour cytotoxicity assay against K562 target cells. The results (expressed as mean percentage cytotoxicity  $\pm$  SEM) are shown in Figure 4.

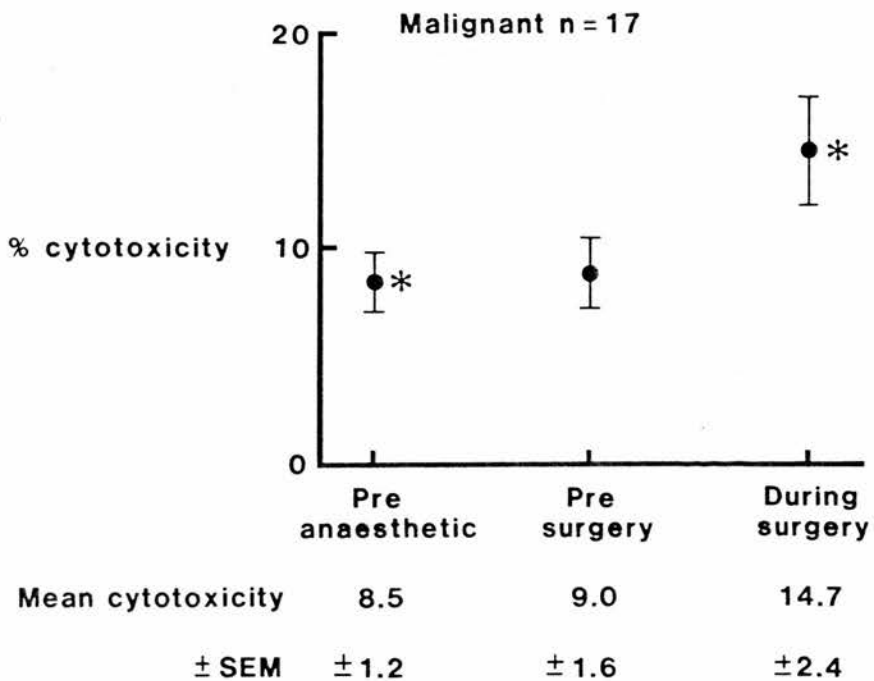
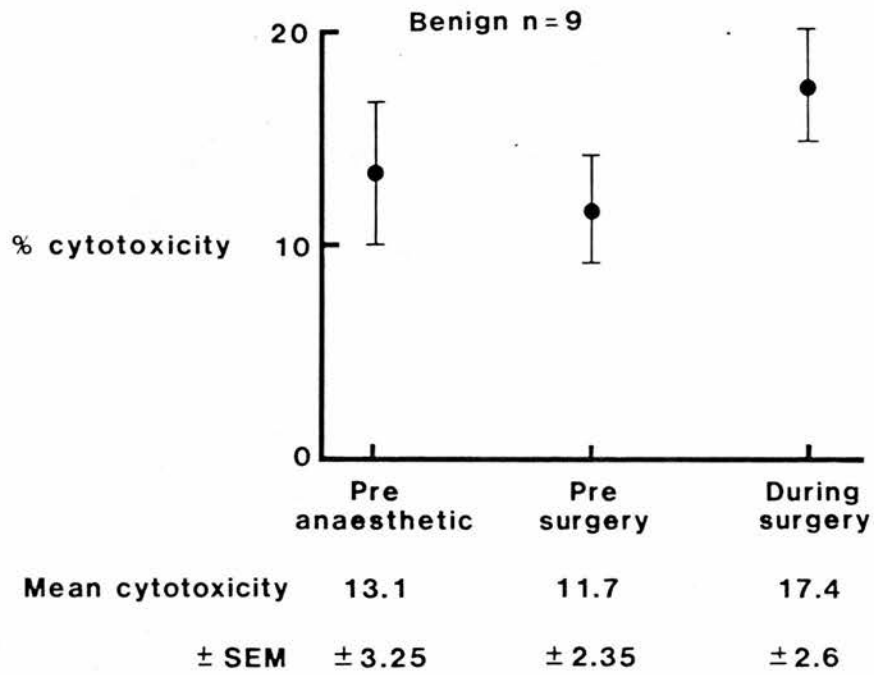
Considering the 9 patients with benign abdominal disease, there was a slight fall in mean  $\pm$  SEM percentage cytotoxicity with induction of anaesthesia from  $13.1 \pm 3.25$  to  $11.7 \pm 2.35$ ; this difference was not statistically significant. The mean  $\pm$  SEM percentage cytotoxicity rose to  $17.4 \pm 2.6$  during the surgical procedure however, due to the small number of patients studied this rise was not significant.

In the 17 patients studied with cancer of the stomach and large bowel, there was a small but statistically insignificant rise in mean  $\pm$  SEM percentage cytotoxicity after the induction of anaesthesia ( $8.5 \pm 1.2$  to  $9 \pm 1.6$ ) but a highly significant rise ( $p < 0.005$ ) during surgery to  $14.7 \pm 2.4$  per cent.

These results show that natural cytotoxicity mediated by host lymphocytes is enhanced during surgery under general anaesthesia but not anaesthesia alone in patients with benign abdominal disease and cancer of the stomach and large bowel. This enhancement of natural cytotoxicity is therefore likely to be related to the surgical procedure itself.

FIGURE 4

PERCENTAGE CYTOTOXICITY TO K562 TARGETS. BEFORE ANAESTHESIA, DURING ANAESTHESIA AND DURING SURGERY IN PATIENTS WITH BENIGN AND MALIGNANT DISEASE. ( $1/2$  DILUTION OF WHOLE BLOOD)

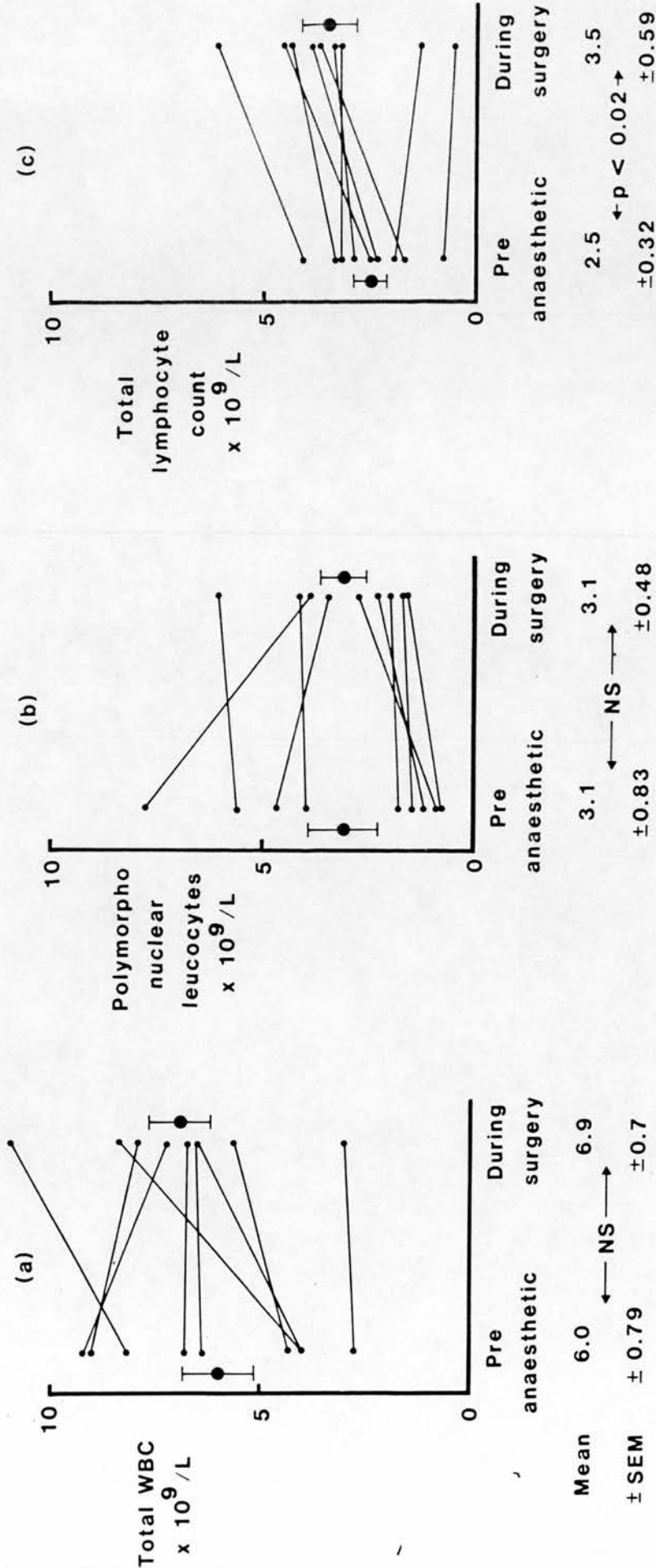


\*  $p < 0.005$

4 hr. chromium release cytotoxicity assay for whole blood against K562 targets.  
 $1/2$  dilution with RPMI.  
 Statistical analysis by paired "t" test.

FIGURE 5

TOTAL WHITE BLOOD CELL COUNT, POLYMORPHONUCLEAR LEUCOCYTE COUNT AND TOTAL LYMPHOCYTE COUNT BEFORE AND DURING SURGERY IN BENIGN PATIENTS (n = 9)



Cell counts by morphological appearances on microscopy after Giemsa staining



5.6 Total white blood cell counts, polymorphonuclear leucocyte counts and total lymphocyte counts before and during surgery in patients with benign disease.

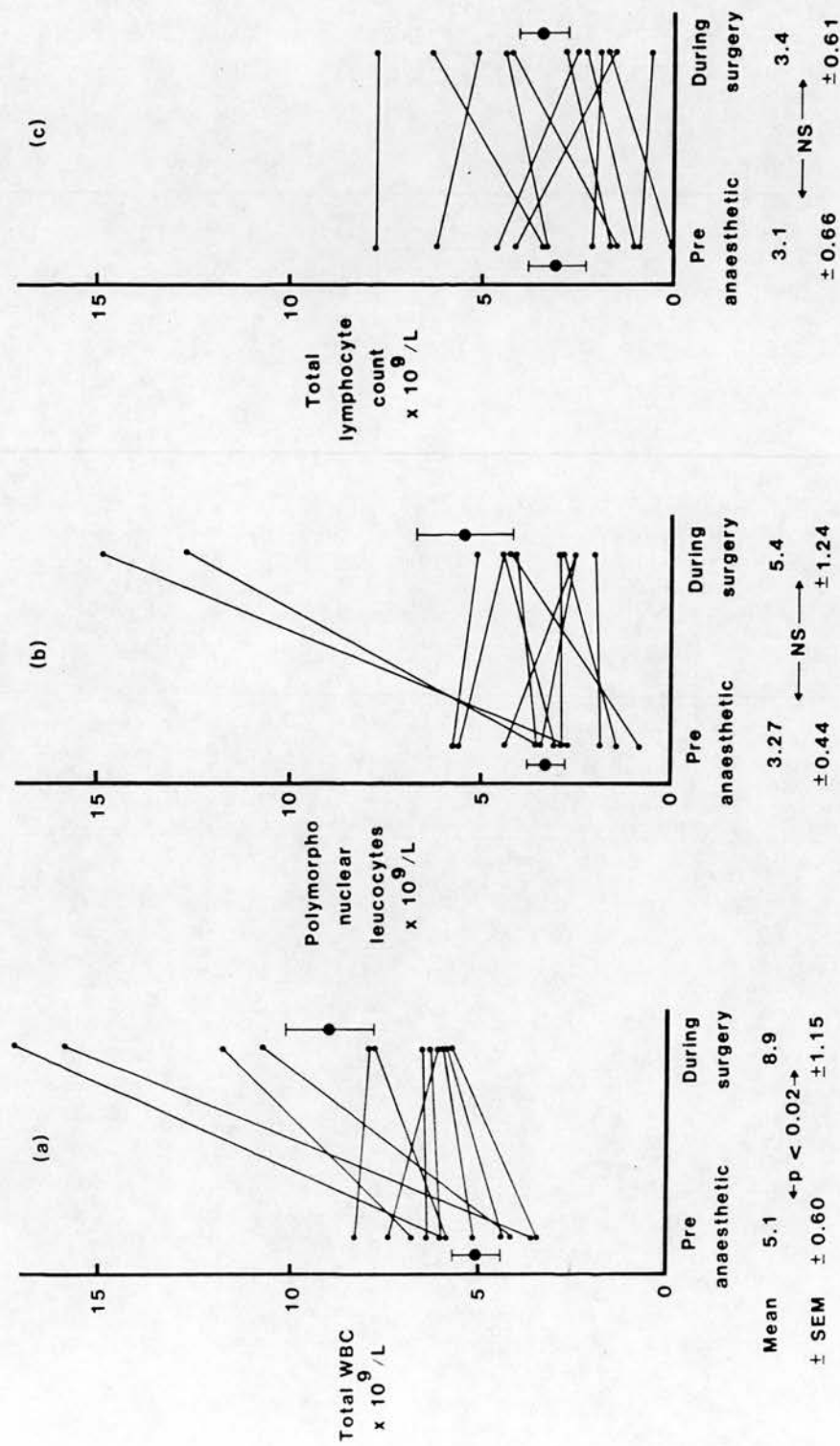
Natural killer lymphocyte activity, as determined by percentage cytotoxicity towards K562 targets, appeared to be enhanced during surgery in patients with benign disease although the mean rise in cytotoxicity just failed to reach statistical significance at the 5% level. A study was undertaken to determine whether the rise in natural cytotoxicity seen during surgical operation was related to an increase in the total white blood cell count of peripheral venous blood taken before and during surgery.

In 9 patients with benign abdominal disease, total white blood cell counts were determined before and during surgery. The results (Fig.5a) showed some individual patient variation with 4 patients showing elevation of total white blood cell count during surgery, 2 showed depression and the remaining 3 patients had no appreciable change in total white cell numbers during surgery. The mean  $\pm$  SEM total white blood cell count rose from  $6.0 \pm 0.79 \times 10^9$  cells/L before surgery to  $6.9 \pm 0.7 \times 10^9$  cells/L during surgery however this rise was not statistically significant.

Polymorphonuclear leucocyte counts (Fig. 5b) were performed before and during surgery, with wide patient variation, but no significant difference in the mean  $\pm$  SEM polymorphonuclear leucocyte count before surgery at  $3.1 \pm 0.83 \times 10^9$  cells/L to that during surgery at  $3.1 \pm 0.48 \times 10^9$  cells/L.

In these patients, however, there was a rise in the mean  $\pm$  SEM total lymphocyte count (Fig.5c) as determined by morphological studies from  $2.5 \pm 0.32 \times 10^9$  cells/L before surgery to  $3.5 \pm 0.59 \times 10^9$  cells/L during surgery. This rise was statistically significant ( $<0.02$ ) by the

**FIGURE 6**  
TOTAL WHITE BLOOD COUNT, POLYMORPHONUCLEAR LEUCOCYTE AND TOTAL LYMPHOCYTE COUNT  
BEFORE AND DURING SURGERY IN MALIGNANT PATIENTS (n = 12)



Cell counts by morphological appearances on microscopy after Giemsa staining

paired "t" test and may account, in part, for the enhanced natural cytotoxicity of blood lymphocytes taken during surgery towards K562 targets.

#### 5.7 Total white blood cell counts, polymorphonuclear leucocyte counts and total lymphocyte counts before and during surgery in patients with cancer.

Studies described previously had shown that in patients with localised malignant disease of the stomach and colon, natural cytotoxicity of lymphocytes towards K562 target cells was enhanced during surgery with general anaesthesia provided either by the inhalational or intravenous route. Total white blood cell counts were performed on 12 patients with malignant disease before and during surgery to determine whether the enhancement of cytotoxicity in these patients was related to an increase in total white blood cell numbers.

The mean  $\pm$  SEM total white blood cell count (Fig.6a) rose from  $5.1 \pm 0.6 \times 10^9$  cells/L before surgery to  $8.9 \pm 1.5 \times 10^9$  cells/L during surgery; this rise was statistically significant ( $<0.02$ ). Four of the 12 patients studied showed a marked increase in total white blood count during surgery which in 2 of these patients resulted in a threefold increase in total white blood cell numbers during surgery. In these 2 patients, the rise in total white blood cells was due to an increase in polymorphonuclear leucocyte numbers (See Fig.6b) however the difference between the mean  $\pm$  SEM polymorphonuclear leucocyte counts before surgery ( $3.27 \pm 0.44 \times 10^9$  cells/L) and during surgery ( $5.4 \pm 1.24 \times 10^9$  cells/L) was not statistically significant.

Five of the 12 cancer patients studied showed a slight rise in total lymphocyte count during surgery (Fig.6c) however the difference between the mean  $\pm$  SEM total lymphocyte count before surgery for all 12 patients, at  $3.1 \pm 0.66 \times 10^9$  cells/L, and the total lymphocyte count during surgery, at  $3.4 \pm 0.61 \times 10^9$  cells/L, was not statistically significant. These results suggest that in the cancer patients studied, the rise in total white blood cell count seen during surgery is accounted for by rises in both polymorphonuclear leucocyte numbers and total lymphocyte numbers during surgery, neither of which was of statistical significance when considered separately.

#### 5.8 Analysis of lymphocyte subpopulations in cancer patients before and during surgery using monoclonal antibody (Leu 7).

The monoclonal antibody (Leu 7) is relatively specific for the natural killer lymphocytes (Abo et al 1980) although it does have a slight cross reactivity with the suppressor lymphocyte population; it was therefore of interest to determine any correlation between the enhanced natural cytotoxic response observed in certain patients and the presence of NK lymphocytes assessed by staining with monoclonal antibody. (Leu 7 monoclonal antibody).

In 11 patients with histologically proven cancer confined to the stomach or large bowel, peripheral venous blood was taken before anaesthesia, and again one hour after induction of anaesthesia during the surgical operation. Estimation of whole blood cytotoxicity to K562 targets was performed using a 4 hour cytotoxicity assay. Peripheral blood lymphocytes were separated from the pre anaesthetic and during surgery blood samples and stained with the monoclonal antibody Leu 7 as described in materials and methods. The number (%) of cells staining were determined by ultra-violet microscopy.

The results of monoclonal antibody staining (as determined by indirect membrane immunofluoresence) are expressed as the percentage of Leu 7 positive cells, together with the cytotoxic activity towards K562 targets during surgery are presented in Table I . Nine of the 11 patients studied showed elevated levels of cytotoxicity to tumour target cells during surgery, 8 of these patients demonstrated a rise in the percentage of Leu 7 positive or NK lymphocytes during surgery and only one patient (JH) with elevated levels of cytotoxicity during surgery showed a slight fall in the percentage of Leu 7 positive cells.

Examining the results from this group of cancer patients as a whole, 9 of the 11 patients showed elevation of natural cytotoxicity during surgery which is likely to be related to the mean  $\pm$  SEM rise in percentage Leu 7 positive cells (NK) during surgery from  $18.4 \pm 2.77\%$  to  $27.4 \pm 2.51\%$ , which represents a significant ( $p < 0.05$ ) rise in Leu 7 positive cells by the paired "t" test.

In the one patient (AH) who had depressed natural cytotoxicity to K562 targets during surgery, the percentage of Leu 7 positive cells was also reduced and in a further patient (HW) although the percentage of Leu 7 positive cells was reduced during surgery, there was no demonstrable effect on percentage cytotoxicity during surgery.

These results suggest that the overall enhancement of natural cytotoxicity to tumour target cells seen during surgery is related to an absolute increase in cell numbers expressing the antigen determinant reactive with Leu 7 monoclonal antibody.



TABLE I

PERCENTAGE OF LEU 7 POSITIVE STAINING CELLS BEFORE  
ANAESTHESIA AND DURING SURGERY IN PATIENTS WITH  
CANCER COMPARED TO THE EFFECT ON CYTOTOXICITY TO  
K562 TARGETS

Patient	Site of tumour	% Leu 7 +ve cells		Cytotoxicity during surgery
		PA	DS	
NW	Colon	12.4	15.8	Elevated
AP	Colon	37.0	33.9	Depressed
FW	Colon	18.0	52.0	Elevated
EM	Rectum	17.0	22.0	Elevated
MS	Colon	7.0	10.0	Elevated
WW	Colon	18.0	41.0	Elevated
JH	Rectum	23.0	21.0	Elevated
AH	Colon	8.3	29.0	Elevated
AC	Stomach	11.0	27.0	Elevated
BR	Stomach	20.0	27.0	Elevated
HW	Stomach	31.0	23.0	No change

Mean	18.4	$p < 0.05$	27.4
		←————→	
± SEM	± 2.77		± 3.51

PA = pre anaesthetic      DS = during surgery

Leu 7 positive staining cells determined by indirect membrane amino fluorescence.

Cytotoxicity of peripheral blood to K562 targets as determined by the 4 hr. chromium release assay.

Statistical analysis by the paired "t" test.



5.9 The percentage of Leu 7 positive cells in the peripheral venous blood pre anaesthesia, pre surgery and during surgery in cancer patients.

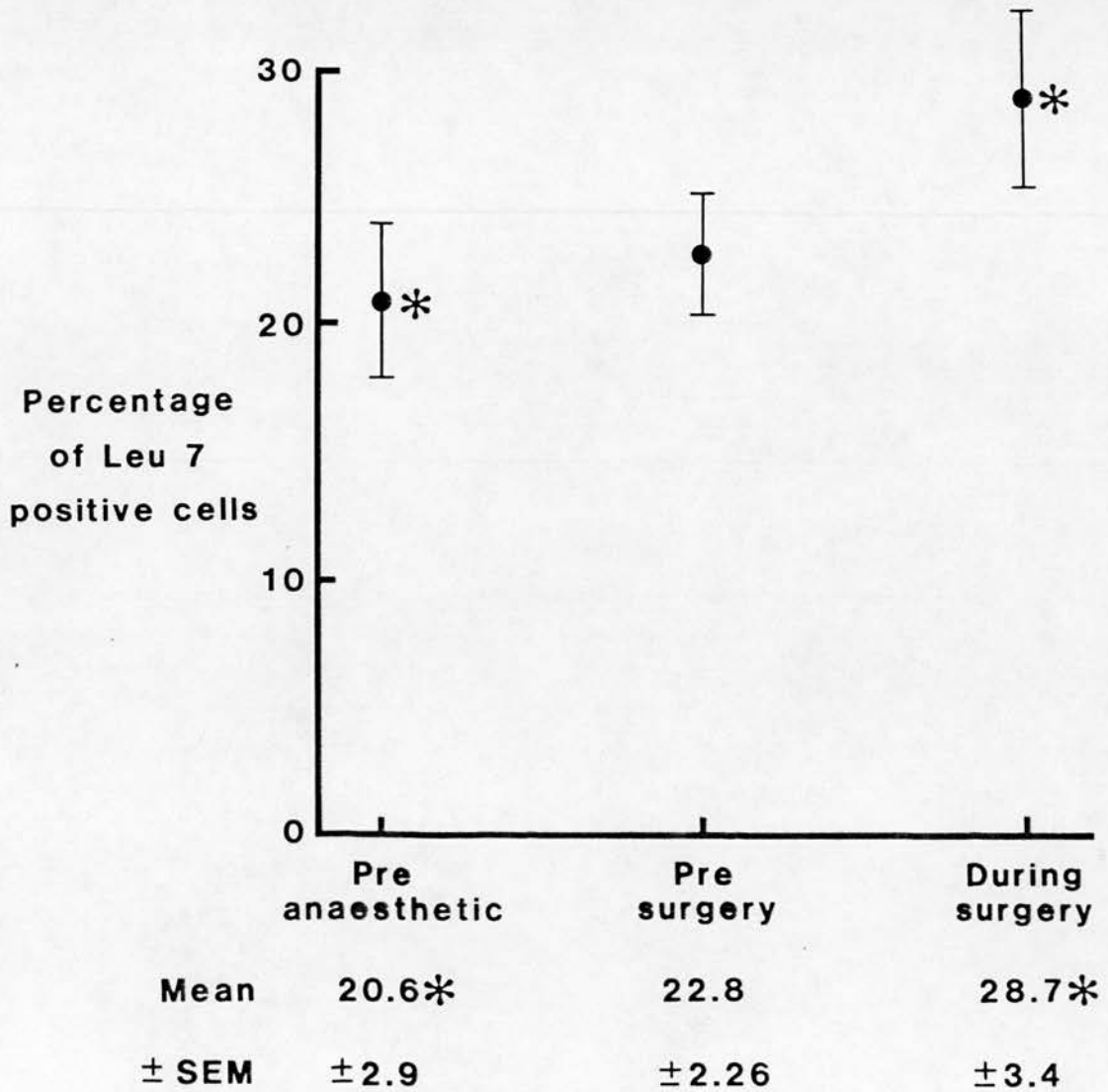
A previous study had shown that the rise in natural cytotoxicity to K562 target cells in patients with cancer did not occur during anaesthesia alone but was evident during surgery undertaken with general anaesthesia, suggesting that the enhancement in natural cytotoxicity is related to the surgical procedure itself.

In this study, whole blood was taken by peripheral venepuncture from 12 patients with localised cancer before induction of anaesthesia, during anaesthesia alone and during surgery. Lymphocytes were separated from the whole blood samples on lymphocyte separation medium and stained with Leu 7 monoclonal antibody in an indirect fluorescent assay prior to examination by ultraviolet microscopy. In figure 7, it can be seen that there is no significant rise between the mean  $\pm$  SEM percentage of Leu 7 positive cells pre anaesthetic ( $20.6 \pm 2.9\%$ ) to the mean  $\pm$  SEM percentage during anaesthesia but before surgery ( $22.8 \pm 2.67\%$ ). There was however, a statistically significant rise ( $p < 0.02$ ) in the mean  $\pm$  SEM percentage of Leu 7 positive cells during surgery ( $28.7 \pm 3.4$ ).

The fact that the rise in percentage Leu 7 positive cells occurs only during surgery under general anaesthesia, but not during anaesthesia alone closely reflects the enhancement of natural cytotoxicity seen in patients with cancer during the surgical procedure under anaesthesia but not under anaesthesia alone. This strongly suggests that enhancement is at least, in part, due to an absolute increase of lymphocytes expressing the Leu 7 antigen marker associated primarily with NK lymphocytes.

**FIGURE 7**

PERCENTAGE OF LEU 7 CELLS BEFORE ANAESTHESIA, DURING ANAESTHESIA ALONE  
AND DURING SURGERY IN CANCER PATIENTS



\*p < 0.02 by paired "t" test

Leu 7 positive cells determined by indirect membrane immuno fluorescence

5.10 Cytotoxicity of whole blood, towards K562 target cells, during surgery and 48 hours following surgery in patients with benign abdominal disease.

In 5 patients with benign abdominal disease undergoing surgery, peripheral venous blood was taken into heparinised tubes before the induction of anaesthesia, during the surgical operation and again 48 hours following surgery. This blood was used in a 4 hour whole blood assay against K562 target cells and the results expressed as percentage cytotoxicity (Table II). Four of the 5 patients showed considerable elevation of percentage cytotoxicity during surgery which was reflected in a significant rise ( $p < 0.02$ ) in mean cytotoxicity from  $14.3 \pm 3.2$  (before induction of anaesthesia) to  $30.8 \pm 4.2$  (during the surgical procedure). Most patients then demonstrated a fall in percentage cytotoxicity 48 hours after surgery with a resultant fall in mean cytotoxicity  $\pm$  SEM to  $17.3 \pm 3.1$ . This activity was not significantly different from the observed pre anaesthetic cytotoxicity for the group ( $14.3 \pm 3.2$ ). These results suggest that natural cytotoxicity of whole blood is elevated during surgery but falls to pre surgery levels within 48 hours of the surgical procedure.

5.11 Cytotoxicity of whole blood, towards K562 target cells, during surgery and 48 hours following surgery in patients with malignant abdominal disease.

In 9 patients with histologically proven malignant disease confined to the stomach or large bowel, peripheral venous blood was taken before the induction of anaesthesia, during the surgical procedure and 48 hours following surgery. This blood was used in a 4 hour cytotoxicity assay

TABLE II

**PERCENTAGE CYTOTOXICITY OF WHOLE BLOOD ( $\frac{1}{2}$  DILUTION)  
FROM PATIENTS WITH BENIGN DISEASE DURING SURGERY AND  
2 DAYS FOLLOWING SURGERY**

Patient	Diagnosis	Pre-anaes.	During surgery	2 days after surgery
KH	Close colostomy	22.5	22.7	25.0
EB	Gallstones	11.4	27.2	17.9
WW	Duodenal ulcer	19.2	33.8	16.7
DS	Close colostomy	4.0	13.5	20.7
GB	Duodenal ulcer	14.3	47.4	6.2
	Mean cytotoxicity	14.3	30.8	17.3
	$\pm$ S.E.M.	$\pm$ 3.2	$\pm$ 4.2	$\pm$ 3.12
P value		<div style="text-align: center;"> <math>p &lt; 0.02</math>  <math>\longleftrightarrow</math>  NS  <math>\longleftrightarrow</math> </div>		

4 hr. chromium release assay for whole blood against K562 targets.

$\frac{1}{2}$  dilution with R.P.M.I.

Statistical analysis by the paired "t" test.

TABLE III

PERCENTAGE CYTOTOXICITY OF WHOLE BLOOD ( $\frac{1}{2}$  DILUTION)  
FROM PATIENTS WITH CANCER DURING SURGERY AND 2 DAYS  
FOLLOWING SURGERY

Patient	Diagnosis	Pre-anaes.	During surgery	2 days after surgery
DG	Ca. colon	11.7	21.9	11.5
JB	Ca. colon	20.8	15.5	17.1
WW	Ca. colon	6.8	18.9	14.1
JC	Ca. colon	26.3	23.4	22.1
RR	Ca. colon	5.8	4.0	5.1
FW	Ca. colon	11.9	19.8	18.7
NF	Ca. colon	16.5	23.7	16.9
DP	Ca. colon	17.5	31.1	15.7
PW	Lymphoma stomach	9.5	30.8	32.7
Mean cytotoxicity		14.0	21.0	17.1
$\pm$ S.E.M.		$\pm 2.25$	$\pm 2.73$	$\pm 2.52$
P value		$\longleftrightarrow$ p < 0.05 $\longrightarrow$		
		$\longleftrightarrow$ NS $\longrightarrow$		

4 hr. chromium release assay for whole blood against K562 targets.

$\frac{1}{2}$  dilution with R.P.M.I.

Statistical analysis by the paired "t" test.

against chromium <sup>51</sup>-labelled K562 target cells, and the results expressed as percentage cytotoxicity (Table III). Six of the 9 patients showed elevation of percentage cytotoxicity during surgery with a statistically significant rise ( $p < 0.05$ ) in mean cytotoxicity  $\pm$  SEM from  $14.0 \pm 2.25$  (pre anaesthesia) to  $21.0 \pm 2.73$  (during the surgical procedure). In 6 patients there was a fall in percentage cytotoxicity 48 hours after surgery with no statistically significant difference between the mean cytotoxicity  $\pm$  SEM in blood taken pre anaesthetic ( $14.0 \pm 2.25$ ) and 48 hours post surgery ( $17.1 \pm 2.52$ ).

This data would suggest that in patients with malignant disease of the stomach and large bowel, natural cytotoxicity to K562 tumour target cells is elevated during the surgical operation and falls towards the pre anaesthetic level in most patients within 48 hours of surgery.

#### 5.12 Plasma interferon levels before and during surgery in patients with benign disease and cancer, demonstrating elevated natural cytotoxicity during surgery.

In 9 patients (3 benign disease, 6 malignant disease) who demonstrated elevated levels of natural cytotoxicity during surgery, plasma was available from the pre surgery and during surgery blood samples, which had been used in the whole blood assay to determine natural cytotoxicity, for the measurement of interferon by the inhibition of virus cytopathic technique described in materials and methods. Interferon is a potent stimulator of antitumour natural killer lymphocyte cytotoxicity (Herberman et al 1979, Karen et al 1981, Stewart et al 1982) and this experiment was conducted to determine whether the rapid production of interferon (IFN) during surgery was related to, and possibly the



mechanism of, elevated natural killer lymphocyte activity which occurred during the surgical operation. It is known that IFN can recruit precursor cells into mature NK lymphocytes as well as enhance the recycling capacity of individual NK cells (Ullberg et al 1981).

The rise in percentage cytotoxicity of the 3 benign and 6 malignant patients during the surgical procedure was highly significant ( $p < 0.001$ ): from  $12.7 \pm 1.58$  to  $26.6 \pm 3.6$  (Table IV). Further tests showed that the rise in percentage cytotoxicity in individual patients during the surgical procedure was not associated with a rise in plasma interferon levels during surgery which in all cases was measured at less than 8.5 IU/ml. In our laboratory, enhancement of natural cytotoxicity of whole blood against K562 targets using the amount of blood used in these experiments (100  $\mu$ L) is consistently found with the addition of exogenous interferon at a concentration of 100 IU/ml. The results in this experiment show that interferon is not produced during the surgical operation and that the levels of interferon detected in the plasma samples were probably too low to account for enhancement of cytotoxicity of the natural killer lymphocytes present in the whole blood samples. It would appear that the enhanced cytotoxic response to K562 targets seen during the surgical procedure in both patients with benign and malignant abdominal disease, is not therefore due to the rapid production of interferon during surgery.

#### 5.13 The effect of exogenous interferon in vitro on natural cytotoxicity towards K562 targets of lymphocytes taken during surgery in patients with benign disease and cancer.

Previous experiments described in this chapter showed that in many patients with benign abdominal disease and cancer of the gastrointestinal

TABLE IV

**PLASMA INTERFERON LEVELS BEFORE AND DURING SURGERY  
IN PATIENTS WITH BENIGN DISEASE AND CANCER**

Patient		Percentage cytotoxicity to K562		Interferon IU/ml	
		PA	DS	PA	DS
WW	(duodenal ulcer)	4.1	33.8	< 8.5	< 8.5
PB	(gallstones)	18.0	45.0	< 8.5	< 8.5
EB	(gallstones)	11.4	27.2	< 8.5	< 8.5
FW	(ca. colon)	11.9	19.8	< 8.5	< 8.5
EM	(ca. rectum)	8.3	14.2	< 8.5	< 8.5
*WD	(ca. colon)	13.3	18.2	< 8.5	< 8.5
BR	(ca. stomach)	19.1	40.4	< 8.5	< 8.5
JH	(ca. rectum)	11.8	17.5	< 8.5	< 8.5
NF	(ca. colon)	16.5	23.7	< 8.5	< 8.5
Mean		12.7	p < 0.001	26.6	
±S.E.M.		±1.58		±3.6	

\*Disseminated disease.

PA = pre anaesthetic      DS = during surgery

4 hr. chromium release cytotoxicity assay of whole blood against K562 targets

½ dilution with RPMI

Interferon measured by inhibition of virus cytopathic effect method

Statistical analysis by paired "t" test

tract, the capacity of natural killer lymphocytes to lyse K562 target cells was enhanced during the surgical procedure. This effect was related to an observed increase in the percentage of Leu 7 positive cells (i.e. mature NK lymphocytes) during surgery. It was of interest therefore to determine whether natural killer lymphocyte ability to kill tumour target cells could be enhanced yet further during the surgical procedure. This could prove beneficial to patients undergoing resection of a malignant tumour when it is known that tumour cells are liberated into the circulating blood, particularly with manipulation of the tumour, with respect to increased tumour cell lysis and prevention of metastatic spread.

In 9 patients, 5 with benign abdominal disease and 4 with gastrointestinal cancer, peripheral venous blood was taken one hour after induction of anaesthesia and start of surgery. Lymphocytes were separated from the whole blood samples as described previously using lymphocyte separation medium, and suspended at a concentration of  $1 \times 10^6$  cells per ml. Lymphocytes were incubated with or without the addition of 100 IU of exogenous human leucocyte  $\alpha$  interferon at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere and subsequently assayed for cytotoxicity against K562 targets in a 4 hour chromium release assay.

The results for the 5 patients with benign disease are shown in Table V. Three patients displayed a significant enhancement ( $p < 0.005$  -  $p < 0.01$  by paired "t" test) of cytotoxicity to K562 tumour targets after incubation of PBL's taken during surgery with interferon. In 2 patients (CS, FI) although there was a rise in cytotoxicity after incubation of PBL's with interferon, this is not statistically significant. Both of these patients PBL's had very high initial levels of cytotoxicity and it may

TABLE V

**THE in vitro EFFECT OF HUMAN LEUCOCYTE  $\alpha$  INTERFERON  
ON PERCENTAGE CYTOTOXICITY OF PBL'S TAKEN FROM  
BENIGN PATIENTS DURING SURGERY**

Patient	Diagnosis	% Cytotoxicity $\pm$ S.E.M. — Inf.	% Cytotoxicity $\pm$ S.E.M. + Inf.	p value
BI	Benign weight loss	38.0 $\pm$ 0.5	60.2 $\pm$ 4.2	<0.01
MS	Gallstones	21.7 $\pm$ 0.8	40.0 $\pm$ 2.1	<0.05
CS	Gallstones	66.2 $\pm$ 0.5	75.9 $\pm$ 10.0	NS
CSt	Gallstones	5.5 $\pm$ 1.1	12.2 $\pm$ 1.6	<0.05
FI	Gallstones	76.6 $\pm$ 4.6	82.4 $\pm$ 1.2	NS

4 hr. chromium release assay for PBL's against K562 targets.

Effector : target ratio 25 : 1

Inf. = Interferon

Statistical analysis by the paired "t" test

TABLE VI

THE *in vitro* EFFECT OF HUMAN LEUCOCYTE  $\alpha$  INTERFERON ON  
PERCENTAGE CYTOTOXICITY OF PBL'S TAKEN FROM CANCER  
PATIENTS DURING SURGERY

Patient	Tumour	% cytotoxicity $\pm$ S.E.M. - Inf.	% cytotoxicity $\pm$ S.E.M. + Inf.	p value
WW	(Ca. colon)	66.5 $\pm$ 3.7	79.0 $\pm$ 5.0	NS
NF	disseminated (Ca. stomach)	0.9 $\pm$ 0.6	2.0 $\pm$ 1.1	NS
PH	(Ca. rectum)	31.3 $\pm$ 4.1	53.0 $\pm$ 2.3	<0.02
FH	advanced (Ca stomach)	45.0 $\pm$ 3.3	70.0 $\pm$ 0.7	<0.01

4 hr. chromium release cytotoxicity assay of PBL's against K562 targets.

Effector : target ratio 25 : 1

Inf. = Interferon

Statistical analysis by paired "t" test

be that they are incapable of further stimulation in terms of increased binding and recycling capacity to result in a significant enhancement of cytotoxicity.

The results for the 4 patients with malignant disease are shown in Table VI. Two patients had tumours localised to the large bowel and two had disseminated gastric cancer. Two patients (PH Ca rectum, FH Ca stomach) displayed significant enhancement ( $31.3 \pm 4.1 - 53 \pm 2.3\%$   $p < 0.02$ ,  $45 \pm 3.3 - 70 \pm 0.7\%$   $p < 0.01$ ) of cytotoxicity to K562 by PBL's taken during surgery after in vitro exposure to interferon. Patient WW did show enhancement of cytotoxicity after exposure of PBL's to interferon ( $66.5 \pm 3.7 - 79 \pm 5.0$ ) but this failed to reach statistical significance by the paired "t" test. Patient NF had widely disseminated gastric cancer with very low levels of cytotoxicity to K562 targets during surgery ( $0.9 \pm 0.6\%$ ) which did not increase significantly after overnight exposure of PBL's to interferon which implies that NK lymphocytes from this patient were refractory to interferon.

These experiments showed that in some patients with cancer, further boosting of already elevated levels of natural cytotoxicity of lymphocytes to tumour target cells can occur following exposure to human leucocyte  $\alpha$  interferon. This could prove of potential therapeutic benefit to patients undergoing resection of a malignant tumour, since enhanced NK cell activity may result in improved lysis of circulating tumour cells and reduction in the likelihood of metastatic tumour formation.

#### 5.14 Discussion.

The experiments described in this chapter show that the antitumour cytotoxic capacity of natural killer lymphocytes, present in circulating



blood is enhanced during the surgical procedure in patients with localised tumours of the stomach and large bowel receiving either inhalational or intravenous anaesthetic agents. Patients with benign disease may also show an enhanced response during surgery; however, patients with advanced malignant disease with tumour metastases failed as a group to show significant enhancement of natural cytotoxicity against K562 tumour target cells during surgery.

This enhancement of natural cytotoxicity seen in patients with localised tumours occurred during the surgical procedure under general anaesthesia but not during anaesthesia alone, in addition the elevated levels of cytotoxicity seen during surgery declined to the pre operative levels within 48 hours following operation in patients with benign abdominal disease and cancer.

The mechanism of this observed enhancement of the cytotoxicity of natural killer lymphocytes towards tumour cells was not the result of a rapid production of interferon during surgery but appeared to be largely due to an increase in absolute numbers of lymphocytes expressing HNK1 antigen (Leu 7) (Abo et al 1980), since Leu 7 monoclonal antibody staining cells were increased in number during surgery. This resulted in an increased number of natural killer lymphocytes present in the circulating blood during the surgical operation, which in patients with cancer could prove to be beneficial, possibly acting on tumour cells liberated from the tumour, particularly during surgical manipulation, and hence improving host defence against potential metastatic spread of the disease. The enhancement of natural cytotoxicity during the surgical procedure in patients with benign abdominal disease occurring in response to stress of the surgical procedure may represent a natural phenomenon necessary

to deal with the potential infectious agents acquired during surgery whilst in the patients with cancer, this defence mechanism is mobilised against liberated tumour cells (Griffith et al 1983). The rise in NK lymphocyte numbers during the surgical procedure probably represents a recruitment response with liberation of NK lymphocytes from precursor cells present in the circulating bone marrow, which results in a temporary redistribution of the NK lymphocytes capable of dealing with the intra operative liberation of tumour cells. This then returns to normal tissue distribution within 48 hours of surgery, resulting in a decline in cytotoxicity to pre operative levels. The failure of patients with metastatic malignancy, and hence large tumour burden, to show elevated levels of natural cytotoxicity to tumour cells during the surgical procedure is perhaps not surprising, since at this stage the tumour may have overwhelmed the host's defence mechanisms against both primary and secondary (metastatic) tumour formation.

The final series of experiments described here show that natural killer lymphocyte activity was enhanced in patients with benign disease and some cancer patients by exposure of blood lymphocytes to exogenous leucocyte  $\alpha$  interferon in vitro. This points to the exciting possibility that in patients with cancer undergoing surgical resection of the tumour, the in vivo use of interferon could be used before surgery to boost natural killer lymphocyte activity of the host. This may then favour enhanced tumour cell lysis during and following the period of surgery when large numbers of tumour cells have been liberated from the tumour. This boosting would facilitate an enhancement of the already elevated natural cytotoxicity occurring during surgery in patients with localised tumours.

Such enhancement activity of the body defences may benefit host survival by increasing the likelihood of lysis of circulating tumour cells with

a potential reduction in tumour metastases seeded at the time of surgical resection of the tumour.

CHAPTER 6.

## DISCUSSION.

### 6.1 Introduction.

Natural killer (NK) lymphocytes have been described in a variety of animal species including man (Rosenberg et al 1972; Kay and Sinkovics 1974; West et al 1977). The natural killer lymphocyte possesses the ability to recognise and cause lysis of abnormal cells such as tumour cells and cells infected by viruses, without the requirements for prior sensitisation to a specific surface antigen (Roder et al 1980). The NK lymphocyte however, does possess a degree of specificity and can recognise macromolecular structures on the surface membranes of susceptible cells which may be transferrin receptor sites (Vodinelick et al 1983); a conjugate is then formed between the NK lymphocyte and the susceptible cell prior to cell lysis (Hiserodt et al 1982). The NK lymphocyte then dissociates from the tumour cell and is available to bind to and kill further tumour cells. The behaviour of NK cells towards abnormal cells, particularly tumour cells, and the fact that they are found in many vertebrate species (rat, hamster, mouse and man) implies that they have a role in primary immune defence against developing tumours (Herberman and Ortaldo 1981) since they can be active within hours of exposure to tumour cells in vivo compared to the time course of 5 to 7 days required for the sensitised cytotoxic T lymphocyte or ADCC to be effective towards and destroy tumour cells (Perrin et al 1977; Zinkernagle and Doherty 1979). NK lymphocytes have also been shown to be active against the haematogenous spread of tumour cells, causing lysis of circulating tumour cells and preventing metastatic tumour formation in mice (Riccardi et al 1979; Hanna and Fidler 1980), and it seems likely that they may have a similar role in defence against secondary haematogenous spread of tumour cells in man.

Patients with immune deficiency syndromes have an increased incidence of malignant disease particularly lymphocyte proliferative disorders, compared to normal controls. Thus, patients with hypogammaglobulinaemia have an increased incidence of leukaemia, those with Chediak Higashi syndrome, in which there is a selective deficit of natural killer lymphocyte activity, are prone to develop lymphoma and those patients receiving high dose immunosuppressive therapy following organ transplantation are more likely to develop lymphoma and epithelial tumours such as skin cancer and cancer of the cervix. These findings would appear to indicate a basic failure of immunological surveillance as pioneered by Burnett.

The NK lymphocyte, because of rapid recognition and subsequent lysis of abnormal cells such as tumour or virus infected cells, has been advocated as a primary mechanism in immune surveillance against developing tumour formation (Greson 1980; Herberman and Ortlando 1981).

## 6.2. Tumour effect on NK lymphocyte activity.

Patients with established malignant disease, whether lymphoma or carcinoma, have been shown by many workers to have depressed NK lymphocyte cytotoxicity to tumour target cells in vitro when compared to non malignant controls (Pross and Baines 1976; Gerson 1980; Hawrylowicz 1982).

The depressed NK lymphocyte activity in patients with malignant disease correlates with the extent of dissemination of the disease or tumour burden rather than the stage of the primary tumour. Patients with early breast cancer were found to have comparable levels of NK lymphocyte activity in the peripheral blood as controls (Eremin 1980), which is similar to the results presented in this thesis, in that patients with



malignant disease localised to the stomach or large bowel had NK activity in peripheral venous blood within the range of patients with benign abdominal disease. The reduction in NK activity of circulating lymphocytes found in patients with disseminated malignancy strongly suggests that the tumour itself has a direct effect on NK lymphocyte activity of the host. These patients could have a defect in immune defence which has allowed the tumour to develop and subsequently consolidate its hold on the host by adversely affecting the host response to the tumour, or alternatively the tumour cells are resistant to NK lysis or cause the production of factors which suppress host natural cytotoxicity.

As further evidence for a direct tumour effect on NK lymphocyte activity, various workers have studied tumour infiltrating lymphocytes (TIL's) for NK mediated cytotoxic effects to tumour target cells. Most workers found either low or absent natural cytotoxicity of TIL's to K562 tumour targets (Vanky and Klein 1982, Bland et al 1981, Vose and Moore 1979) and studies in our laboratory on tumours from patients used in this study have shown that most colorectal tumours have low, but detectable, levels of natural cytotoxicity to K562 target cells. (Clegg and Rees, personal communication). Studies of tumour draining lymph nodes in patients with breast cancer (Eremin 1980) or large bowel cancer (Vose et al 1980) have shown low or absent natural cytotoxicity of lymphocytes in these nodes suggesting a direct tumour effect on these cells. Low levels of natural cytotoxicity in tumour infiltrating and nodal lymphocytes by possible modulation of NK cell activity by the tumour may be a mechanism for continued growth and spread of malignant tumours.

Possible reasons for depressed natural killer cell activity from intratumour lymphocytes are that there are low numbers of effector cells within the tumour or that the tumour infiltrating lymphocytes with natural killer cell activity are present but their activity is inhibited by tumour or host cell derived factors.

Introna et al (1982) have suggested that low intratumour NK activity could be due to low numbers of effector cells. This is not supported by experiments from workers in our laboratory who have shown that many colorectal tumours have low but significant levels of NK cytotoxicity to K562 target cells. Similarly histochemical staining using the Leu 7 monoclonal antibody which identifies NK lymphocytes have shown substantial numbers of lymphocytes with a positive staining reaction with Leu 7 antibody (Fig.1.) in colorectal cancers and breast cancers (Rooney 1983, personal communication) and Leu 7 positive cells in colorectal tumour digests account for about 8% of the tumour infiltrating host cell population.

The experiments reported in this thesis with the Leu 7 labelling of lymphocytes in the arterial blood entering the tumour and the venous blood draining the tumour have shown no gross change in the NK cell (Leu 7 positive) population entering and leaving the tumour circulation. This suggests that a steady state situation exists with respect to NK lymphocytes in the blood passing through the tumour circulation and the intratumour NK lymphocytes, and that net sequestration of NK lymphocytes in the tumour does not occur. Further evidence that low intratumour NK lymphocyte activity is not due to low numbers of effectors comes from Golub et al (1982) who has shown with the single cell assay that depressed NK activity of tumour infiltrating lymphocytes is due to the presence of suppressive factors within the tumour.

FIGURE 1.



SECTION OF A COLONIC ADENOCARCINOMA SHOWING LEU 7 POSITIVE (NK) LYMPHOCYTES  
IN THE TUMOUR SUBSTANCE.

(Histochemical staining technique combined with Leu 7 antibody makes Leu 7  
positive cells appear brown - courtesy of Dr. N. Rooney).

NK lymphocyte activity in established malignant tumours may be suppressed directly by tumour products or activation of host cells such as macrophages or lymphocytes by the tumour which then produce factors which directly suppress NK activity.

Malignant tumours are known to produce hormones and other biologically active products which are related to the lineage of the malignant cell, however, some tumour products seem to be able to directly influence the host response to the tumour with marked depression of that response.

Serum from patients with cancer inhibited the cytotoxicity of peripheral blood T lymphocytes to tumour target cells (Nind et al 1975) and serum from patients with advanced gastric carcinoma induced suppressor cell activity in lymphocytes which then caused inhibition of lymphocyte transformation (Toge et al 1983).

Edwards et al (1973) showed that a factor present in the blood directly draining gastrointestinal tract tumours caused direct inhibition of lymphocyte transformation, and in the present study tumour draining plasma components were shown to depress NK cytotoxicity of PBL's from normal individuals. Nair et al (1980) have demonstrated inhibition of both NK and ADCC in mice with cancer sera and natural killer lymphocyte cytotoxicity to K562 target cells was substantially reduced by incubation with the supernatant from tissue culture of human hepatic cancer cells (Keony et al 1983). The nature of the inhibitory effect of the tumour product was postulated as blocking the receptor sites of the NK effectors thus reducing binding to target cells. The results presented in this thesis confirm the likely presence of a plasma factor which is in high concentration in the blood draining malignant tumours of the stomach and colon, which causes significant depression of

natural killer lymphocyte activity from normal controls. This factor is probably heat stable but further work is required to determine the nature of this factor, its molecular weight and chemical structure and also the cell responsible for producing the factor i.e. either the tumour cells per se or intratumour lymphocytes or macrophages. It is of considerable interest that Flannery has reported a heat stable, pH resistant substance of low molecular weight produced by macrophages which inhibit NK lymphocyte activity in mice (1983 personal communication), and further studies should be initiated to determine whether intratumour macrophages from human tumours release suppressor substances.

Circulating immune complexes have frequently been found in the blood of patients with cancer (Day et al 1982, Hoffken et al 1977, Theofilopoulos et al 1977, Bonavida and Zighelboim 1974) with high levels often relating to poor patient prognosis. These complexes probably represent excess tumour antigen which is complexed with host antibody. It would seem reasonable to implicate immune complexes as a possible mechanism for NK lymphocyte inhibition by binding to the Fc receptor present on the NK lymphocyte. Studies by Timonen and Saksela (1977) have shown that purified monoclonal IgM blocks NK cell activity and NK inhibition by IgG containing complexes acts by preventing binding of effector cells to target cells.

The results of the studies presented in this thesis show that tumour draining venous plasma which caused significant depression of NK lymphocyte activity from normal controls possessed very low levels of immune complexes as detected by the Raji cell assay. Although this assay is not infallible in detecting circulating immune complexes, it would be expected that if this was a likely mechanism for the depression



of NK lymphocyte activity, raised levels of immune complexes would be found in at least some of the patients included in the study; this however was not shown.

Suppressor cells either by direct cell to cell contact or release of suppressor substances may be responsible for depression of natural killer lymphocyte activity in cancer patients.

Zoller et al (1982) found that cells recovered from Percoll gradient separation and present in the Percoll fraction 1.090 to 1.121 g/ml were effective in the suppression of natural killer, K (killer) cell and cytotoxic T cell function in mice with cell to cell contact required for suppression. Eremin (1980) has found tumour associated lymphocytes from patients with breast cancer caused inhibition of NK lymphocyte activity and other workers (Vanky and Klein 1982) have suggested the existence of nylon wool adherent tumour infiltrating cells in colorectal cancer which inhibit natural killing of TIL's.

Adherent monoclonal cells (macrophages) from malignant pleural effusions were found by Uchida and Michsche (1981) to suppress NK lymphocyte cytotoxicity to tumour target cells in vitro. These studies suggest that suppressor cells, either tumour associated lymphocytes or macrophages, probably release suppressor substances which cause inhibition of the NK lymphocyte's capacity to kill tumour cells. Cudkowicz and Hochman (1979) reported that supernatants from cultured suppressor cells from mouse spleens inhibited NK lymphocyte cytotoxicity in mice, and Flannery (1983, personal communication) has reported a low molecular weight product from mouse macrophages which inhibits NK cell activity.



Further studies with isolation of tumour associated lymphocyte and macrophages by currently available techniques from human cancers will help to identify the cell population responsible for NK lymphocyte depression in man. It is well established that prostaglandins which inhibit NK cell function are produced both by tumours (Bennett et al 1977, 1982) and host mononuclear phagocytes (Doller et al 1978). Further studies are required to identify the role of prostaglandins in NK lymphocyte depression of the host.

### 6.3 The effects of anaesthesia and surgery on the immune response with particular reference to the natural killer lymphocyte.

Metastatic spread of malignant tumours remain the major problem in the treatment of malignant disease in man. From studies on experimental animals, tumour cells are found in the circulation when blood vessels of a given size (30µm) penetrate the growing tumour allowing tumour cells either individually or in clumps to escape (Liotta et al 1976). Circulating tumour cells are found in cancer patients which may lodge in capillary networks and lie dormant for many years before forming overt metastases (Fisher and Fisher 1967). These authors state that "all tumour emboli may represent potential metastases" therefore their presence in the circulating blood of patients with cancer must be taken as evidence for the metastatic potential of the primary tumour. The immune depression seen during anaesthesia and surgery which affects both the humoral and cell mediated immune response has been implicated in the dissemination of malignant disease following resection of the primary tumour (Salo 1982).

There is no generalised agreement as to whether it is the effects of anaesthesia or the surgical procedure itself that causes depression of in vitro immune responses. Of the inhalational anaesthetic agents, halothane is associated with in vitro depression of lymphocyte cytotoxicity (Cullen et al 1976) and inhibition of lymphocyte motility (Nunn et al 1970) whilst nitrous oxide also inhibits cell mediated cytotoxicity (Cullen et al 1976) and prolonged administration is toxic to bone marrow (Bruce 1980), which is the source of NK cell precursors.

The surgical procedure was considered by Riddle (1967) and Park et al (1971) to be the major cause of depression of in vitro lymphocyte responses and studies by Berenbaum et al (1973) and Cullen and Van Belle (1975) report that the lymphocyte depression following surgery was directly related to the extent of surgical trauma.

In patients undergoing donor nephrectomy, for transplant purposes who are free from malignant disease, there is a decrease in the number of circulating T and B lymphocytes following surgery (Slade et al 1975) which may also pertain for patients with malignant disease. Patients with cancer have a reduced T cell response to mitogens during surgery (Cullen and Van Belle 1975) and an attenuated response to antigen stimulation (Cochran et al 1972). Cytotoxicity of lymphocytes to autologous tumour cells is decreased post operatively in patients with breast cancer (Vose and Mougdil 1975), malignant melanoma (Cochran et al 1972) and antibody dependent cytotoxicity or K cell activity is decreased following operation in previously immuno suppressed cancer patients (McCredie et al 1979).

There have been no published reports about NK lymphocyte activity during anaesthesia and surgery in patients with cancer or benign disease

apart from the author's (Griffith et al 1983a, Griffith et al 1983b, Griffith et al 1983c) but other workers have found that natural killer lymphocyte cytotoxicity to tumour target cells is depressed when measured from the third post operative day in patients with breast cancer (Uchida et al 1982). A study of patients with solid tumours also showed depression of natural killer lymphocyte activity 4 to 6 weeks following surgery (Flad et al 1983) however it must be stressed that neither of these studies measured NK lymphocyte cytotoxicity to tumour target cells during the surgical procedure. The results presented in this thesis show that the capacity of natural killer lymphocytes to kill tumour target cells (K562) is enhanced during the surgical operation in both patients with benign abdominal conditions and tumours localised to the stomach or large bowel, but patients with disseminated malignancy do not show such marked enhancement. This effect occurs during the surgical procedure under anaesthesia (with both intravenous and inhalational anaesthetic agents) but not anaesthesia alone, and appears to be due to an increase in cells bearing the monoclonal antibody marker for NK lymphocytes (Leu 7) during the surgical procedure. This could be seen as a recruitment response with the mobilisation of NK lymphocyte precursors from bone marrow to cope with the likely events during the surgical procedure such as liberation of tumour cells by manipulation of the tumour. This recruitment response would ultimately lead to exhaustion of the bone marrow-derived NK precursors which probably is reflected in the return to pre operation levels of NK lymphocyte mediated cytotoxicity of the patients studied within 48 hours of the surgical procedure, and to the post surgical depression of NK cytotoxicity observed by other workers.

Surgical resection of the primary tumour under general anaesthesia offers the best chance of cure of gastrointestinal cancer but it is known that

malignant cells from the primary tumour are present in the circulating blood before and during the surgical procedure (Engell 1955, Salisbury et al 1965). These circulating tumour cells are viable and can be grown in tissue culture (Moore et al 1958).

Manipulation of the tumour during surgery greatly increases the number of circulating malignant cells (Griffith 1960) which coupled with a degree of depression of host immune defences during anaesthesia and surgery, may combine to produce a fertile "soil" for the seeding of tumour cells to form potential metastatic tumour deposits.

There is abundant evidence from experimental animals of increased tumour growth and dissemination of tumour with the formation of metastases following anaesthesia and surgery. Grottfried et al (1961) described an increase in the growth rate of implanted tumours after repeated surgical wounding and El Rifi et al (1965) has reported an increased incidence of pulmonary metastases following laparotomy in rats; the formation of metastases was counteracted by heparin which presumably prevented implantation of malignant cells and thus allowed host defence mechanism to destroy circulating tumour cells.

The evidence for increased dissemination of malignant disease in man following surgical operation is sparse but anecdotal reports in the literature have suggested rapid growth and metastatic spread of primary tumours after resection of the tumour or incidental minor surgery. (Gordon Taylor 1948, Lewis and Cole 1958, Jewell and Romsdahl 1965).

In the context therefore of immune suppression of conventional T and B lymphocyte responses during surgical resection of primary malignant tumours, it is encouraging to find that at least one potential mechanism

for defence against intravascular dissemination of the tumour, namely the natural killer lymphocyte, is enhanced during the surgical procedure itself and remains enhanced for up to 48 hours post operatively. The fact that the already enhanced cytotoxicity of human NK lymphocytes to tumour target cells due to an increase in cell numbers in the circulation can be further enhanced in vitro by exposure to interferon leads to the exciting possibility of selective immunological enhancement of NK lymphocyte activity in vivo with infusion of interferon pre-operatively. This could lead to improved lysis of circulating tumour cells and a possible reduction or abolition of metastases developing following surgery. This concept should be evaluated by future research using a controlled animal model system using a spontaneously metastasizing tumour which parallels the behaviour of human cancers.

There are many clinical trials which have now been conducted that show NK lymphocyte activity can be boosted in vivo with interferon given by the intramuscular or intravenous route (Bordeau et al 1982 ; Quesada et al 1982; Einhoru et al 1982; Lucero et al 1982). The enhancement occurs 24 to 36 hours after exposure to interferon and declines to pre exposure levels within 1 to 3 weeks. It is also of interest that whilst the NK lymphocyte is very efficient at killing cells from the K562 erythroleukaemia cell line, activity against autologous tumour cells from the same patients may be low yet this can be considerably improved by exposure to interferon in vivo (Mantovani et al 1983), and similarly Rees et al (1983) have shown considerable enhancement of NK mediated lysis of human ocular malignant melanoma cells in vitro after exposure to exogenous interferon.

There is therefore, a potential therapeutic application projected from the results presented in this thesis that pre operative stimulation of the host natural killer lymphocyte response by infusion of interferon 24 to 36 hours before surgery, could be used as a manoeuvre to increase killing of circulating tumour cells during and for up to 48 hours after the surgical resection of the primary tumour. This may reduce or abolish the formation of metastases following surgery by preventing the implantation of circulating malignant cells which have the potential to become metastatic tumour deposits.

### 6.3 Proposed future research.

The natural killer lymphocyte may represent an effective defence against the metastatic spread of a tumour which is already established in the host and research into the possible tumour directed mechanisms for depression of activity of these natural killer lymphocytes may yield an increased understanding of the mechanisms of tumour suppression of host defences. I have already evidence of a tumour associated plasma factor which causes suppression of NK lymphocyte activity in vitro. This may be produced by the tumour cells or intra tumour host cells such as macrophages or lymphocytes which have been directed by the tumour to produce inhibitory substances. Isolation of tumour infiltrating cells by currently available techniques could help to identify which cell population is responsible for the depressant factor.

Another part of my current research has shown that natural killer lymphocyte activity in cancer patients can be stimulated by biological response modifiers such as interferon. Studies of interferon given in vivo to patients with cancer have shown enhancement of natural killer



cell activity 24 to 48 hours following injection. This phenomenon could be used to advantage in patients undergoing resectional surgery for cancer. Surgical manipulation of a tumour releases tumour cells which are found in the circulating blood and with the generalised immunosuppression associated with general anaesthesia and surgery, these cells could form potential metastases. Enhancement of a defence mechanism against tumour metastases such as the natural killer lymphocyte system during the perioperative period when the patient is at risk of implicating tumour cells to form potential metastases, could prove beneficial in terms of improved patient survival. A clinical trial of selective NK lymphocyte enhancement by agents such as interferon used in the perioperative period in patients undergoing potentially curative surgery for gastrointestinal tract cancer should be undertaken.

## REFERENCES.

REFERENCES.

ABO T, BALCH C.M.

A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1).

J. Immunol. 1981, 127, 1024-1029

BAKER C.C, MILLER C.L, TRUNKEY D.D. et al

Identity of mononuclear cells which compromise the resistance of trauma patients.

J. Surg. Res. 1979, 26, 478-487

BECKER S.

Intratumour NK reactivity.

In: Natural Cell Mediated Immunity Against Tumours.

Ed: R.B. Herberman, Academic Press, New York. 1980, 985-996

BENNETT A, CARROLL M. A, STAMFORD I. F. et al

Prostaglandins and human lung carcinomas.

Br. J. Cancer. 1982, 46, 888-893

BENNETT A, CHARLIER E. M, McDONALD A.M. et al

Prostaglandins and breast cancer.

Lancet. 1977, 2, 624-626

BERENBAUM M. C, FLUCK P. A, HURST N. P.

Depression of lymphocyte responses after surgical trauma.

Br. J. Exp. Pathol. 1973, 54, 597-607

BERGHEDEN C, HELLSTRÖM K. E.

Studies on allogenic inhibition. II. Inhibition of mouse tumour cell growth by in vitro contact with cells containing foreign H-2 antigens. Int. J. Cancer. 1966, 1, 361-369

BLAND P. W, BRITTON D. C, RICHENS E. R. PLEDGER J. V.

Peripheral, mucosal and tumour infiltrating components of cellular immunity in cancer of the large bowel. Gut. 1981, 22, 744-751

BONAVIDA B, ZIGHELBOIM J.

Modulation of the immune response towards allografts in vivo.  
I. Selective suppression of the development of cell mediated immunity by soluble alloantigens. Cell Immunol. 1974, 13, 52-65

BORDEN E. C, HOLLAND J. F, DAO T. L. et al

Leukocyte derived interferon (alpha) in human breast carcinoma. The American Cancer Society Phase II trial. Ann. Intern. Med. 1982, 97, 1-6

BOYSE E. A.

Immune responses to experimental tumours. Guy's Hosp. Rep. 1963, 112, 433-448

BOYUM A.

Separation of leucocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. 21 (Suppl. 97) 1968, 77-89

BRUCE D. L.

Anaesthesia, formed elements of the blood and macrophages.

Federation Proceedings. 1980, 39, 1592-1594

BRUNNER K. T, MAVEL J, CEROTTINI J. C. CHAPUIS B.

Quantitative assay of the lytic action of immune lymphoid cells on <sup>51</sup>Cr labelled allogenic target cells in vitro. Inhibition by isoantibody and by drugs.

Immunol. 1968, 14, 181-195

BRUNDA M. J, HERBERMAN R. B, HOLDEN H. T.

Interferon independent activation of murine natural killer cell activity.

In: Natural Cell Mediated Immunity Against Tumours.

Ed: R.B. Herberman, Academic Press, New York. 1980, 525-536

BURNET F. M.

The concept of immunological surveillance.

Progr. Exp. Tumour Res. 1970, 13, 1-27

CLEGG A, REES R. C.

1983, Personal communication.

COCHRAN A.J, MACKIE R. M, THOMAS C. E, GRANT R. M, CAMERON-MOWAT D. E.

SPLIG W. G. S.

Cellular immunity to breast carcinoma and malignant melanoma.

Br. J. Cancer. 1973, 28, Suppl.1, 77-82

COCHRAN A. J, SPLIG W. G. S, MACKIE R. M. et al

Post operative depression of tumour directed cell mediated immunity  
in patients with malignant disease.

B.M.J. 1972, 4, 67-70

CUDKOWICZ G, HOCHMAN P. S.

Do natural killer cells engage in regulated reactions against self to  
ensure homeostasis?

Immunol. Rev. 1979, 44, 13-41

CULLEN B. F, DUNCAN P. G, RAYKIEL L.

Inhibition of cell mediated cytotoxicity by Halothane and Nitrous Oxide.  
Anesthesiology. 1976, 5, 386-390

CULLEN B. F, VAN BELLE G.

Lymphocyte transformation and changes in leucocyte count: Effects of  
anaesthesia and operation.

Anesthesiology. 1975, 43, 563-569

DAY N. K, GOOD R. A, WITKIN S. S.

Circulating immune complexes and complement in human malignancy.

In: Immune Complexes and Plasma Exchanges in Cancer Patients.

Ed: B. Serou and C. Rosenfeld. Elsevier Biomedical Press, Holland.

1981, 99-108

DJEU J. Y, HEINBAUGH J. A, HOLDEN H. T. et al

Role of macrophages in the augmentation of mouse natural killer cell  
activity by poly I:C and interferon.

J. Immunol. 1979, 122, 182-188



DJEU J. Y, TIMONEN T, HERBERMAN R. B.

Augmentation of natural killer cell activity and induction of interferon by tumour cells and other biological response modifiers. In: Symposium on the Role of Natural Killer Cells, Macrophages and Antibody Dependent Cellular Cytotoxicity in Tumour Rejection and as Mediators of Biological Response Modifiers Activity.

Ed: M.A. Chivigos, Raven, New York. 1981

DOHERTY P.C, GÖTZE R, TRINCHIERI G, ZINKERNAGEL R.M.

Models for recognition of virally modified cells by immune thymus derived lymphocytes.

Immunogenetics. 1976, 3, 517-524

DULBELLO D et al

Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. 1954, 99, 167-182

EDWARDS A. J, ROWLAND G. F, LEE M. R.

Reduction of lymphocyte transformation by a factor produced by gastrointestinal cancer.

Lancet. 1973, 1, 687-689

ELRIFI K, BACON B, MEHIGAN J, HOPPE E, COLE W. H.

Increased incidence of pulmonary metastases after celiotomy: Counteraction by heparin.

Arch. Surg. 1965, 91, 625-629

ENGELL H. C.

Cancer cells in the circulating blood.

Acta Chir. Scand. (Suppl.) 1955, 201, 1-70

EREMIN O.

NK cell activity in the blood, tumour draining lymph nodes and primary tumours of women with mammary carcinoma.

In: Natural Cell Mediated Immunity Against Tumours.

Ed: R.B. Herberman. Academic Press, New York. 1980, 1011-1029

EVERSON T. C, COLE W.

Spontaneous regression of cancer.

Ed: T.C. Everson, W. Cole.

Philadelphia and London. Saunders. 1966.

FINSTAD J, GOOD R. A.

Phylogenetic studies of adaptive immune responses in the lower vertebrates.

In: Phylogeny of Immunity.

Ed: R.A. Good. University of Florida Press, Gainesville. 1966, 173-189

FISHER E. R. FISHER B.

Recent observations on concepts of metastases.

Arch. Path. 1967, 83, 321-324

FLAD H. D, BETZLER M, SCHOLZ W. et al

Modulation of NK cell activity in patients with solid malignancies by surgical tumour reduction and fibroblast interferon.

In: Natural Killer and Other Natural Effector Cells.

Ed: R. B. Herberman, Academic Press, New York. 1983, 195-204

FLANNERY G.

1983. Personal communication

FOLEY E. J.

Antigenic properties of methylcholanthrene induced tumours in mice of the strain of origin.

Cancer Res. 1953, 13, 835-837

FREEDMAN V. H, BROWN A. L, KLINGER H. P. et al

Mass production of animal cells in nude mice with retention of cell specific markers.

Exp. Cell. Res. 1976, 98, 143-151

GERSON J. M.

Systemic and in situ natural killer activity in tumour bearing mice and patients with cancer.

In: Natural Cell Mediated Immunity Against Tumours.

Ed: R.B. Herberman, Academic Press, New York. 1980, 1047-1062

GOLDFARB R H, HERBERMAN R. B.

The role of neutral serine proteases in the mechanism of tumour cell lysis by human natural killer cells.

In: NK Cells and Other Natural Effector Cells.

Ed: R.B. Herberman, Academic Press, New York. 1982, 931-938

GOLUB S. H, NITSUMA M, KAWATZ N. et al

NK activity of tumour infiltrating and lymph node lymphocytes in human pulmonary tumors.

In: NK Cells and Other Natural Effector Cells.

Ed: R.B. Herberman, Academic Press, New York. 1982, 1113-1118

GORDON-TAYLOR G.

On Cancer of the breast.

Ann. R. Coll. Surg. 1948, 2, 60-67

GORDON-TAYLOR G.

The incompatible factor in cancer prognosis.

B.M.J. 1959, 1, 455-462

GORELICK E, HERBERMAN R. B.

Radioisotope assay for evaluation of in vivo natural cell-mediated resistance of mice to local transplantation of tumour cells.

Int. J. Cancer. 1981, 27, 847-856

GOTTFREID B, MOLOMUT N, SKAREDOFF L.

Effect of repeated surgical wounding on the growth rate of a tumour graft.

Ann. Surg. 1961, 153, 138-142

GRESSER I.

Antitumour effects of interferon.

In: Cancer, A Comprehensive Treatise.

Ed: F.F. Becker, Academic Press, New York. 1977

GRIFFITH C. D. M, REES R. C, PLATTS A. et al (a)

The effect of anaesthesia and surgery on natural killer lymphocytes in patients with cancer and benign disease (Abstract)

Clinical Oncology 1984. In Press.

GRIFFITH C. D. M, REES R. C, PLATTS A. et al (b)

Enhanced natural killer cell activity in patients with malignant disease of the stomach and large bowel during intravenous anaesthesia and surgery. Preliminary Report.

Journal of Exp. and Clin. Canc. Res. 1983, 2, 399-402

GRIFFITH C. D. M, REES R. C, PLATTS A. et al (c)  
 Enhancement of natural killer cell activity during surgery. Further  
 enhancement in vitro with exogenous interferon. (Abstract).  
 Clinical Oncology. 1984. In Press.

GRIFFITHS J. D.

The dissemination of cancer cells during operative procedures.  
 Ann. Roy. Coll. Surg. Eng. 1960, 27, 14-44

GROSS L.

Intradermal immunization of C3H mice against a sarcoma that originated  
 in animal of the same line.  
 Cancer Res. 1943, 13, 835-837

HALLER O, KIESSLING R, ORN A. et al

Generation of natural killer cells : an autonomous function of the  
 bone marrow.  
 J. Exp. Med. 1977, 145, 1411-1416

HANNA N, FIDLER I. J.

Role of natural killer cells in the destruction of circulating  
 tumour emboli.  
 J. Nat. Cancer Inst. 1980, 65, 801-809

HAWRYLOWICK C. H, REES R. C, POTTER C. W. HANCOCK S. W.

Altered natural killer cell response in patients with malignant lymphoma.  
 Phil. Trans. R. Soc. Lond. 1982, B299, 125-144

HELLSTROM I.

A colony inhibition (Cl) technique for demonstration of tumour cell destruction by lymphoid cells in vitro.

Int. J. Cancer. 1967, 2, 65-68

HERBERMAN R. B.

Natural killer (NK) cells.

In: The Lymphocyte.

Ed: Alan R. Liss, New York. 1981, 33-43

HERBERMAN R. B, HOLDEN H. T.

Natural cell-mediated immunity.

Adv. Cancer Res. 1978, 27, 305-377

HERBERMAN R. B, ORTALDO J. R.

Natural killer cells : Their role in defenses against disease.

Science. 1981, 214, 24-30

HERBERMAN R. B, ORTALDO J. R, BONNARD G. D.

Augmentation by interferon of human natural and antibody dependent cell mediated cytotoxicity.

Nature 1979, 277, 221-223

HERON I, BERG K, CANTELL K.

Regulatory effect of interferon on T cells in vitro.

J. Immunol. 1976, 117, 1370-1373



HERSEY P, EDWARDS A, MCCARTHY W. H.

Tumour related changes in natural killer cell activity in melanoma patients. Influence of stage of disease, tumour thickness and age of patients.

Int. J. Cancer. 1980, 25, 187-194

HERSEY P, EDWARDS A, MCCARTHY W. et al

Tumour related changes and prognostic significance of natural killer cell activity in melanoma patients.

In: NK Cells and Other Natural Effector Cells.

Ed: R. B. Herberman, Academic Press, New York. 1982, 1167-1174

HEWITT H. B.

Animal tumour models and their relevance to human tumour immunology.

Journal of Biological Response Modifiers. 1982, 1, 107-119

HEWITT H. B, BLAKE E. R, WALDER A. S.

A critique of the evidence for active host defence against cancer based on the personal studies of 27 murine tumours of spontaneous origin.

Brit. J. Cancer. 1976, 33, 241-259

HISERODT J. C, BRITVAN L. J, TARGAN S. R.

Characterisation of the cytolytic reaction mechanism of the human natural killer (NK) lymphocyte, resolution into binding, programming and killer cell independent steps.

The Journal of Immunology. 1982, 129, 1782-1787

HOBBISS J, COOPER K. M, MOORE M. et al

Limitations of immune complex measurements in colorectal disease.

Br. J. Surg. 1983, 70, 473-477

HOCHMAN P. S, LUDKOWICZ G.

Suppression of natural cytotoxicity by spleen cells of hydrocortisone treated mice.

J. Immunol. 1979, 123, 968-976

HOFFKEN K, MEREDITH I. D, ROBINS R. A. et al

Circulating immune complexes in patients with breast cancer.

B.M.J. 1977, 2, 218-220

HOLLINSHEAD A. C, STEWART T. H. M, HERBERMAN R. B.

Dealted hypersensitivity reaction to soluble membrane antigens of human malignant lung cells.

J. Nat. Cancer Inst. 1974, 52, 327-338

HUDIG D, HAVERTY T, FULCHER D. et al

~~Limitations of human natural cytotoxicity by macromolecular antiproteases.~~

J. Immunol. 1981, 126, 1569-1574

INTRONA M, ALLAVENA P, ACERO T. et al

NK cell activity in human ovarian tumours.

In: NK Cells and Other Natural Effector Cells.

Ed: R.B. Herberman, Academic Press, New York. 1982, 1119-1126

ISAACS A, LINDENMAN K.

Virus interference. I. The Interferons.

Proc. Roy. Soc. (London) 1957, 147, 258-267

JEWELL W. R, ROMSDHAL M. M.

Recurrent malignant disease in operative wounds not due to surgical implantation from the resected tumour.

Surgery. 1965, 58, 806-809

JOHNSTON M. D.

The characteristics required for a Sendai preparation to induce high levels of interferon in human lymphoblastoid cells.

Journal. Gen. Virol. 1981, 56, 175-184

JONES D, LAURENCE A. S, THORNTON J. A.

Total intravenous anaesthesia with etomidate - fentanyl.

Anaesthesia. 1983, 38, 29-34

JONDAL M, TARGAN S.

In vitro induction of cytotoxic effector cells with spontaneous killer cell activity.

J. Exp. Med. 1978, 147, 1621-1636

KADISH A. S, DOYLE A. T, STENHAUER E. H, GHOSSEIN N. A.

Natural cytotoxicity and interferon production in human cancer: Deficient natural killer activity and normal interferon production in patients with advanced disease.

J. Immunol. 1981, 127, 1817-1823

KAPLAN J, CALLEWAERT D. M.

Expression of human T-lymphocyte antigens by natural killer cells.

J. Nat. Cancer Inst. 1978, 60, 961-964

KARRE K, KELIN G. O, KIESSLING R. et al

Low natural in vivo resistance to syngenic leukaemias in natural killer deficient mice.

Nature. 1980, 284, 624-626

KAY D. H, SINKOVICS J. G.

Cytotoxic lymphocytes from normal donors.

Lancet. 1974, 2, 296-297

KENNEDY M. S, STOBO J. D, COLDYNE M. E.

In vitro synthesis of prostaglandins and related lipids by populations of human peripheral blood monocytes.

Prostaglandins 1980, 20, 135-145

KEONG A, HERMAN J, ROBSON A.R.

Supernatant derived from a human hepatocellular carcinoma cell line (PLC/PRF/5) depresses natural killer (NK) cell activity.

Cancer Immunol. Immunother. 1983, 15, 183-187

KLEIN G.

Tumour antigens.

Annual Rev. Microbiol. 1966, 20, 223-252

KLEIN G, SJÖGREN H. O, KLEIN E, HELLSTRÖM E.

Demonstration of resistance against methylcholanthrene - induced sarcomas in the primary autochthonous host.

Cancer Res. 1960, 20, 1561-1572

KOIDE Y, TAKASUGI M.

Determination of specificity in natural cell mediated cytotoxicity by natural antibodies.

J. Nat. Cancer Inst. 1977, 59, 1099-1105

KOREN H. S, ANDERSON S. J, FISCHER D. G. et al

Regulation of human natural killing. I. The role of monocytes, interferon and prostaglandins.

J. Immunol. 1981, 127, 2007-2013

KURLAND J. I, BOCKMAN R.

Prostaglandin E production by human blood monocytes and mouse peritoneal macrophages.

J. Exp. Med. 1978, 147, 952-957

LEWIS M. R, COLE W. H.

Experimental increase of lung metastases after operative trauma (amputation of limb with tumour).

Arch. Surg. 1958, 77, 621-626

LEWIS M. G, IKONOPISOV R. L, NAIRN R. C, PHILLIPS T. M,

HAMILTON FAIRLEY G, BODENHAM D. C, ALEXANDER P.

Tumour specific antibodies in human malignant melanoma and their relationship to the extent of the disease.

B.M.J. 1969, 3, 547-552

LIOTTA L. A, KLEINERMAN J, SAIDEL G M.

The significance of haematogenous tumour cell clumps in the metastatic process.

Cancer Res. 1976, 36, 889-894

LOZZIO G. B, LOZZIO B. B.

Human chronic myelogenous leukaemia cell line with positive Philadelphia chromosome.

Blood. 1975, 45, 321-334

LUCERO M. A, MAGDELENAT H, FRIDMAN W. H. et al

Comparison of effects of leucocyte and fibroblast interferon on immunological parameters in cancer patients.

Eur. J. Cancer Clin. Oncol. 1982, 18, 243-251

MACLEAN L. D.

Host resistance in surgical patients.

Journal of Trauma. 1979, 19, 297-304

MANTOVANI A, ALLEVENA P, BIONDI A. et al

NK activity in human ovarian carcinoma.

In: NK cells and other natural effector cells.

Ed: R.B. Herberman. Academic Press, New York, 1982, 123-137

MARCHALONIS J. J, EDELMAN G. M.

Phylogenetic origins of antibody structure. III. Antibodies in the primary immune response of the sea lamprey. *Petromyzon marinus*.

J. Exp. Med. 1968, 127, 891-914



McCREDIE J. A, MACKDONALD H. R, WOOD S. B.

Effect of operation and radiotherapy on antibody dependent cellular cytotoxicity.

Cancer. 1979, 44, 99-105

MIDDLE J. G, EMBLETON M. J.

Naturally arising tumours of the inbred WAB/NOT rat strain.

J. Nat. Cancer Inst. 1981, 67, 637-643

MINTZ B, SILVERS W. K.

Intrinsic immunological tolerance in allophenic mice.

Science. 1967, 158, 1484-1487

MOLLER G.

Effect on tumour growth in syngeneic recipients of antibodies against tumour specific antigens in methylcholanthrene induced mouse sarcomas.

Nature. 1964, 204, 846-847

MOORE G. E, MOUNT D. T, WENDT A. C.

The growth of human tumour cells in tissue culture.

Surgical Forum Clinical Congress. 1958, 9, 572-576

NAIR P. N. M, FERNANDES G, ONOE K. et al

Inhibition of effector cell functions in natural killer cell activity (NK) and antibody dependent cellular cytotoxicity (ADCC) in mice by normal and cancer sera.

Int. J. Cancer. 1980, 25, 667-677

NIND A. P. P, MATTHEWS N, PIHL E. A. U. et al.

Analysis of inhibition of lymphocyte cytotoxicity in human colon carcinoma.

Br. J. Cancer. 1975, 31, 620-629

NUNN J. F, SHARP J. A, KIMBALL K. L.

Reversible effect of an inhalational anaesthetic on lymphocyte motility.

Nature. 1970, 226, 85-86

OEHLER J. R, LINDAY C. R, NUNN M. E, HOLDEN H. T, HERBERMAN R. B.

Natural cell mediated cytotoxicity in rats. In vivo augmentation of NK cell activity.

Int. J. Cancer. 1978, 21, 210-220

OJO E. WIGZELL H.

Natural killer cells may be the only cells in normal mouse lymphoid cell populations endowed with cytolytic ability for antibody coated tumour target cells.

Scan. J. Immunol. 1978, 7, 297-306

O'TOOLE C, PERLMANN P, WIGZELL H, UNSGAARD B, ZETTERLUND C G.

Lymphocyte cytotoxicity in bladder cancer. No requirement for thymus derived effector cell?

Lancet. 1973, 1, 1085-1088

PARK S. K, BORDY J. I, WALLACE H. A. et al

Immunosuppressive effect of surgery.

Lancet 1971, 1, 53-55

PERRIN C H, ZINKERNAGEL R. M, OLDSTONE M. B.

Immune response in humans after vaccination with vaccinia virus:  
generation of a virus specific cytotoxic activity by human peripheral  
lymphocytes.

L. Exp. Med. 1977, 146, 949-969

PETER H. H, PAVIE -FISCHER J, FRIDMAN W. J. et al

Cell mediated cytotoxicity in vitro of human lymphocytes against a  
tissue culture melanoma cell line. (1GR3).

J. Immunol. 1975, 115, 539-548

PREHN R. T, MAIN J. M.

Immunity to methylolanthrene - induced sarcomas.

J. Nat. Cancer Inst. 1957, 18, 768-778

PRICE M. R, BALDWIN R. W.

Preparation of aminoazo dye induced rat hepatoma membrane fractions  
retaining tumour specific antigen.

Brit. J. Cancer. 1974, 30, 382-393

PROSS H. F. BAINES M. G.

Spontaneous human lymphocyte mediated cytotoxicity against tumour  
target cells. I. The effect of malignant disease.

Int. J. Cancer. 1976, 18, 593-604

PROSS H. F, BAINES M.G.

Studies of human natural killer cells. I. In vivo parameters affecting  
normal cytotoxic functions.

Int. J. Cancer. 1982, 29, 383-390

PROSS H. F, BAINES M. G, JONDAL M.

Spontaneous human lymphocyte mediated cytotoxicity against tumour target cells. II. Is the complement receptor necessarily present on the killer cells?

Int. J. Cancer. 1977, 20, 353-358

QUESADA J. R, GUTTERMAN J. U, HERSH E.M.

Clinical and immunological study of beta interferon by intramuscular route in patients with metastatic breast cancer.

J. Interferon Res. 1982, 2, 593-599

RABINOVITCH M, MANEJIAS R. E, RUSSO M. et al

Increased spreading of macrophages from mice treated with interferon inducers.

Cell Immunol. 1977, 29, 86-95

REES R. C, PLATTS A. A.

A modified short term cytotoxicity test: Assessment of natural cell mediated cytotoxicity in whole blood.

J. Immunol. Methods. 1983, 62, 79-85

REES R. C, RENNIE I. G, PLATTS A. et al

Susceptibility of human ocular melanoma cells to spontaneous and interferon augmented natural cytotoxicity.

Cancer.Letters. 1983, 18, 205-213

RICCARDI C, PUCCETTI P, SANTONI A, HERBERMAN R. B.

Rapid in vivo assay of mouse natural killer cell activity.

J. Nat. Cancer Inst. 1979, 63, 1041-1045

RIDDLE P. R.

Disturbed immune reactions following surgery.

Br. J. Surg. 1967, 54, 882-886

RITCHIE A. W. S, OSWALD I, MICKLEM H. S. et al

Circadian variation of lymphocyte subpopulations : a study with monoclonal antibodies.

B.M.J. 1983, 286, 1773-1775

ROBERTS S, LONG L, JONASSON O, McGRATH R, McGREW E, COLE W. H.

The isolation of cancer cells from the blood stream during uterine curettage.

Surg. Gyn. Obstet. 1960, 111, 3-11

RODER J. C, ARGOV S, KLEIN M. et al

Target effector cell interaction in the natural killer cell system.

V. Energy requirements, membrane integrity and the possible involvement of lysosomal enzymes.

Immunology. 1980, 40, 107-116

RODER J, DUWE A.

The beige mutation in the mouse selectivity impairs natural killer cell function.

Nature. 1979, 278, 451-453

RODER J. C, HELFAND S. L, WERKMEISTER J, McGARRY R, NEAUMONT T. J, DUWE A.

Oxygen intermediates are triggered early in the cytolytic pathway of human NK cells.

Nature 1982, 298, 560-572

ROONEY N.

1983. Personal communication.

ROSENBERG E. B, HERBERMAN R. B, LEVINE P. H, HALTERMAN R. H,  
McLOY J. C, WUNDERLICH J. R.

Lymphocyte cytotoxicity reactions to leukaemia associated antigens  
in identical twins.

Int. J. Cancer. 1972, 9, 648-658

ROSENBERG E. B, McCOY J. L, GREEN S. J. et al

Destruction of human lymphoid tissue culture lines by human  
peripheral lymphocytes in <sup>51</sup>Cr release cellular cytotoxicity assays.

J. Nat. Cancer Inst. 1974, 52, 345-352

RYGAARD J, POVLSEN C. O.

The nude mouse vs the hypothesis of immunological surveillance.

Transplant Rev. 1976, 28, 43-61

SALIMONU L. S, OJO-AMAIZE E, WILLIAMS A. I. O, JOHNSON A. O. K,  
COOKE A. R, ADEKUNLE F. A, ALM G. V, WIGZELL H.

Depressed natural killer cell activity of children with protein calorie  
malnutrition.

Clinical Immunology and Immunopathology. 1982, 24, 1-7

SALSBURY A. J, McKINNA J. A, GRIFFITH J. D, NAUTON MORGAN C.

Circulating cancer cells during excision of carcinomas of the rectum  
and colon with high ligation of the inferior mesenteric vein.

Surg. Gyn. Obstet. 1965, 120, 1266-1269



SALO M.

Trauma, stress and immunity in anaesthesia and surgery.

Eds: Watkins and Salo. Butterworth Scientific, London 1982, 211

SALOMEN P.

Increased tumour metastases from subcutaneous primary tumour in NK deficient (beige) mice.

Proc. Eortc. Conf. on Metastases. 1980, London. Abs. 34.

SANTONI A, HERBERMAN R. B, HOLDEN H. T.

Correlation between natural and antibody dependent cell mediated cytotoxicity against tumour targets in the mouse. II. Characterisation of the effector cells.

J.N.C.I. 1979, 63, 995-1003

SAVARY C. A, LOTZOVA E.

Suppression of natural killer cell cytotoxicity by splenocytes from *Corynebacterium parvum* injected, bone marrow tolerant and infant mice.

J. Immunol. 1978, 120, 239-243

SHANG WANG B, HENCOCK E. H, MANNICK J. A.

Characterisation of suppressor cells generated in mice after surgical trauma.

Clinical Immunology and Immunopathology. 1982, 24, 161-170

SHIKU H, TAKAHASHI T, CAREY T. et al

Definition of cell surface antigens of human malignant melanoma, astrocytoma and renal cancer by typing with autologous serum

In: Serologic Analysis of Human Cancer Antigens.

Ed: S. Rosenberg, Academic Press, 1980, 305-337

SLADE M. S, SIMMONS R. L, YUNIS E. et al

Immunodepression after major surgery in normal patients.

Surgery. 1975, 78, 363-371

STEINHAEUER E. H, DOYLE A T, REED J. et al

Defective natural cytotoxicity in patients with cancer: Normal number of effector cells but decreased recycling capacity in patients with advanced disease.

The Journal of Immunology 1982, 129, 2255-1159

STEWART L. D, HINSON A, ADES E. W.

Enhanced binding and functional activity of human natural killer cells after interferon treatment.

Clinical Immunology and Immunopathology. 1982, 25, 133-138

STUTMAN O.

Chemical carcinogenesis in nude mice: comparison between nude mice from homozygous matings and heterozygous matings and effect of age and carcinogen dose.

J. Nat. Cancer Int. 1979, 62, 353-358

TAKASUGI M, KLEIN E.

A microassay for cell mediated immunity.

Transpl. 1970, 9, 219-227

TAKASUGI M, MICKEY M. R, TERASAKI P. I.

Reactivity of lymphocytes from normal persons on cultured tumour cells.

Cancer Res. 1973, 33, 2898-2902

THEOFILOPOULOS A. N, WILSON C. B, DIXON F. J.

The Raji cell radioimmune assay for detecting immune complexes in human sera.

J. Clin. Invest. 1976, 57, 169-182

THEOFILOPOULOS A. N, ANDREWS B. S, URIST M. M. et al

The nature of immune complexes in human cancer sera.

J. Immunol. 1977, 119, 657-663

THOMAS L.

Cellular and humoral aspects of the hypersensitive state.

Ed: H. Lawrence, Hocker, New York, 1959, 529

TIMONEN T, ORTALDO J. R, HERBERMAN R. B.

Characteristics of human large granular lymphocytes and relationship to natural killer and K cells.

J. Exp. Med. 1981, 153, 569-582

TIMONEN T, SAKSELA E.

Human natural cell mediated cytotoxicity against fetal fibroblasts.

I. General characteristics of the cytotoxic activity.

Cell Immunol. 1977, 33, 340-352

TOGE I, TANADA M, YAJIMA K. et al

Induction of suppressor cell activity in normal lymphocytes by sera from gastric cancer patients.

Clin. Exp. Immunol. 1983, 54, 80-86

UCHIDA A, KOLB R, MICHSCHKE M.

Generation of suppressor cells for natural killer activity in cancer patients after surgery.

J. Nat. Cancer Inst. 1982, 68, 735-741

UCHIDA A, MICHSCHKE M.

Natural killer cells in carcinomatous pleural effusions.

Cancer Immunol Immunother. 1981, 11, 131-138

ULLBERG M, MERRILL J, JONDAL M.

Interferon - induced NK augmentation in humans. An analysis of target recognition effector cell recruitment and effector cell recycling.

Scand. J. Immunol. 1981, 14, 285-293

VANKY F, ARGOV S.

Human tumour - lymphocyte interferon in vitro VII. Blastogenesis and generation of cytotoxicity against autologous tumour biopsy cells are inhibited by interferon.

Int. J. Cancer. 1980, 26, 405-411

VANKY F, KLEIN E.

Autologous lytic potential of lymphocytes separated from human solid tumours.

In: NK Cells and Other Natural Effector Cells.

Ed: R.B. Herberman, Academic Press, New York, 1982, 1061-1068

VONDINELICH L, SUTHERLAND R, SCHNEIDER C. et al

Receptor for transferrin may be a "target" structure for natural killer cells.

Proc. Natl. Acad. Sci. U.S.A. 1983, 80, 835-839

VOSE B. M.

Natural killers in human cancers: Activity of tumour infiltrating and draining node lymphocytes.

In: Natural Cell Mediated Immunity Against Tumours.

Ed: R.B. Herberman, Academic Press, New York, 1980, 1081-1097

VOSE B. M, MOORE M.

Suppressor cell activity of lymphocytes infiltrating human lung and breast tumours.

Int. J. Cancer. 1979, 24, 579-585

VOSE B. M, MOORE M.

Natural cytotoxicity in humans. Susceptibility of freshly isolated tumour cells to lysis.

J. Nat. Cancer Inst. 1980, 65, 257-263

VOSE B. M, MOUGDIL G. C .

Effect of surgery on tumour directed leucocyte responses.

B.M.J. 1975, 1, 56-58

VOSE B. M, VANKY F, FORD M, KELIN E.

In vitro generation of cytotoxicity against autologous human tumour biopsy cells.

Int. J. Cancer. 1978, 21, 588-593

VOSE B. M, VANKY GF, KLEIN E.

Human tumour lymphocyte interaction in vitro. Comparison of reactivity of tumour infiltrating, blood and lymph node lymphocytes with autologous tumour cells.

Int. J. Cancer. 1977, 20, 895-902

WELLS A, BURDICH J. F, CHRISTIANSEN C. et al

Demonstration of tumour associated delayed cutaneous hypersensitivity reactions in patients with lung cancer and in patients with carcinoma of the cervix.

Natl. Cancer Inst. Mong. 1973, 37, 197-203

WEST W. H, CANNON G. B, KAY M. K. et al

Natural cytotoxic reactivity of human lymphocytes against a myeloid cell line. Characterization of effector cells.

J. Immunol. 1977, 118, 355-361

WHITE D, JONES B, COOKE T. et al

Natural killer (NK) activity in peripheral blood lymphocytes of patients with benign and malignant breast disease.

Br. J. Cancer. 1982, 46, 611-616

WOOD S.

Pathogenesis of metastasis formation observed in the rabbit ear chamber.

Arch. Pathol. 1958, 66, 550-568

ZARLING J. M, SCHLAIS J, ESKRA L. et al

Augmentation of human natural killer cell activity by polyinosinic acid - polycytidylic acid and non toxic mismatched analogues.

J. Immunol. 1980, 124, 1852-1857



ZINKERNAGEL R. M, DOHERTY P. C.

MHC restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T cell restriction, specificity, function and responsiveness.

Adv. Immunol. 1979, 27, 51-177

ZOLLER M, MATZKU S, ANDRIGHETTO G. et al

Regulation of NK reactivity by suppressor cells.

In: NK cell and Other Natural Effector Cells.

Ed: R.B. Herberman, Academic Press, New York, 1982, 541-548

PUBLICATIONS.Publications from the work presented in this thesis:

Enhanced natural killer cell activity in patients with malignant disease during intravenous anaesthesia.

C.D.M. Griffith, R.C. Rees, A. Platts, A.S. Laurence, K. Rogers.

The Journal of Experimental and Clinical Cancer Research, 1983, 2, 399-402

The behaviour of natural killer lymphocytes from peripheral blood and spleen in patient with malignant disease.

C. Harowitz, S. Ali, C.D.M. Griffith, R.C. Rees.

The Journal of Cancer Research and Clinical Oncology, 1983, 106, 202-209

The nature of enhanced natural killer cell activity in patients with benign disease and cancer during anaesthesia and surgery.

C.D.M. Griffith, R.C. Rees, A. Platts, A. Jermy, K. Rogers.

Annals of Surgery (In Press)

Modulation of natural killer cell cytotoxicity by tumours of the gastrointestinal tract (Abstract)

C.D.M. Griffith, R.C. Rees, A. Platts, K. Rogers.

British Journal of Surgery, 1983, 70, 695

Enhanced natural killer cell activity during anaesthesia and surgery in patients with malignant disease (Abstract)

C.D.M. Griffith, R.C. Rees, A. Platts, K. Rogers.

Clinical Oncology, 1984 (In Press)

In preparation:

Modulation of natural killer cell activity by gastrointestinal tumours.

C.D.M. Griffith, R.C. Rees, A. Platts, C. Penning, K. Rogers.